Development of Amphibian-based Models of Thyroid-axis Disruption

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McKim Conference

June 2006

Duluth, MN

Preview

- Why is EPA working on thyroid axis
- Thyroid axis summary
- Amphibian metamorphosis assays
- In vitro assays
- High-throughput assays for QSAR modeling

U.S. Law Requires EPA to Evaluate Chemicals for Their Potential to Affect Endocrine Function

- Food Quality Protection Act
    - estrogens or other endocrine effects
- Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC)
- Estrogens, androgens, and thyroid hormone

Why Thyroid Hormone?

- **Important for vertebrate growth and development**
  - Protection of infants and children
  - Neurodevelopment
  - Reproduction

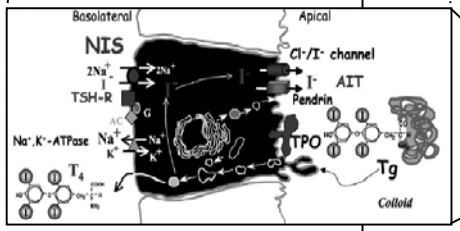
The Challenge Facing EPA

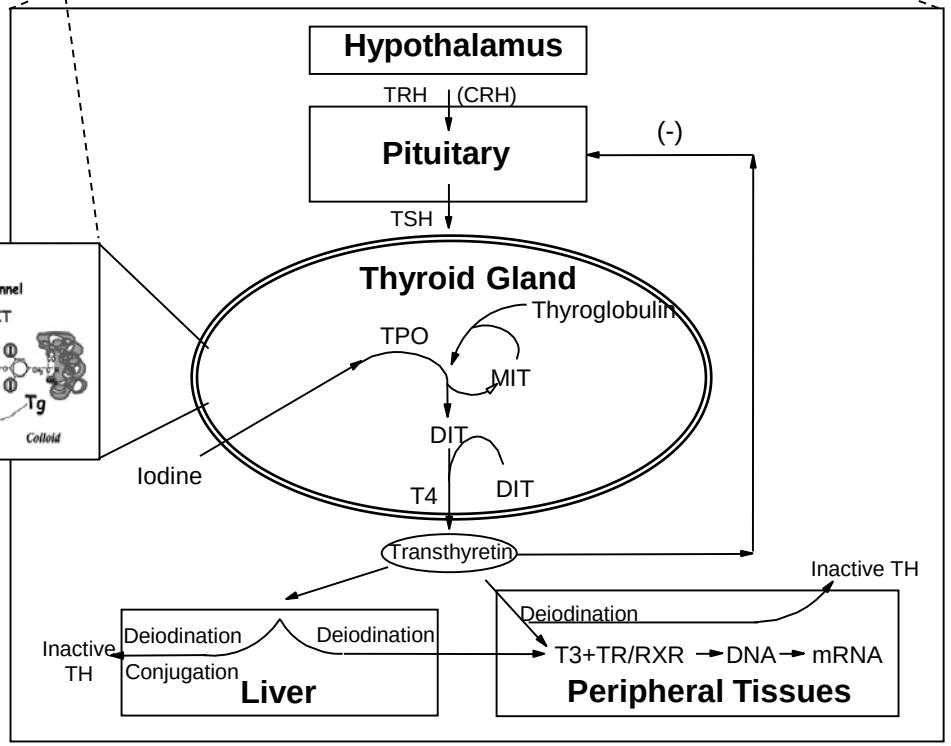
- Ecological and Human Health Risk Assessments for chemicals seek to anticipate and limit adverse outcomes
  - Proactive vs Reactive
- Challenges:
  - Limited data
    - Pre-Manufacture Notifications ~ 2500 new chemicals/yr
  - Large number of chemicals
    - TSCA Industrial Inventory >70,000 chemicals
      - 90 day turn around
    - Pesticide Inerts
      - Which ~800 need tolerances determined by ’06
    - EDCs; (FQPA)
      - >6000 chemicals need to be prioritized

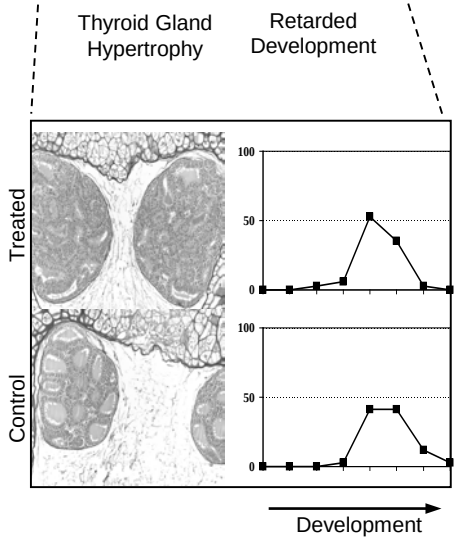
Thyroid-axis Systems Model

QSAR and *in vitro* Models → **Systems Model** → **Organismal Outcomes**

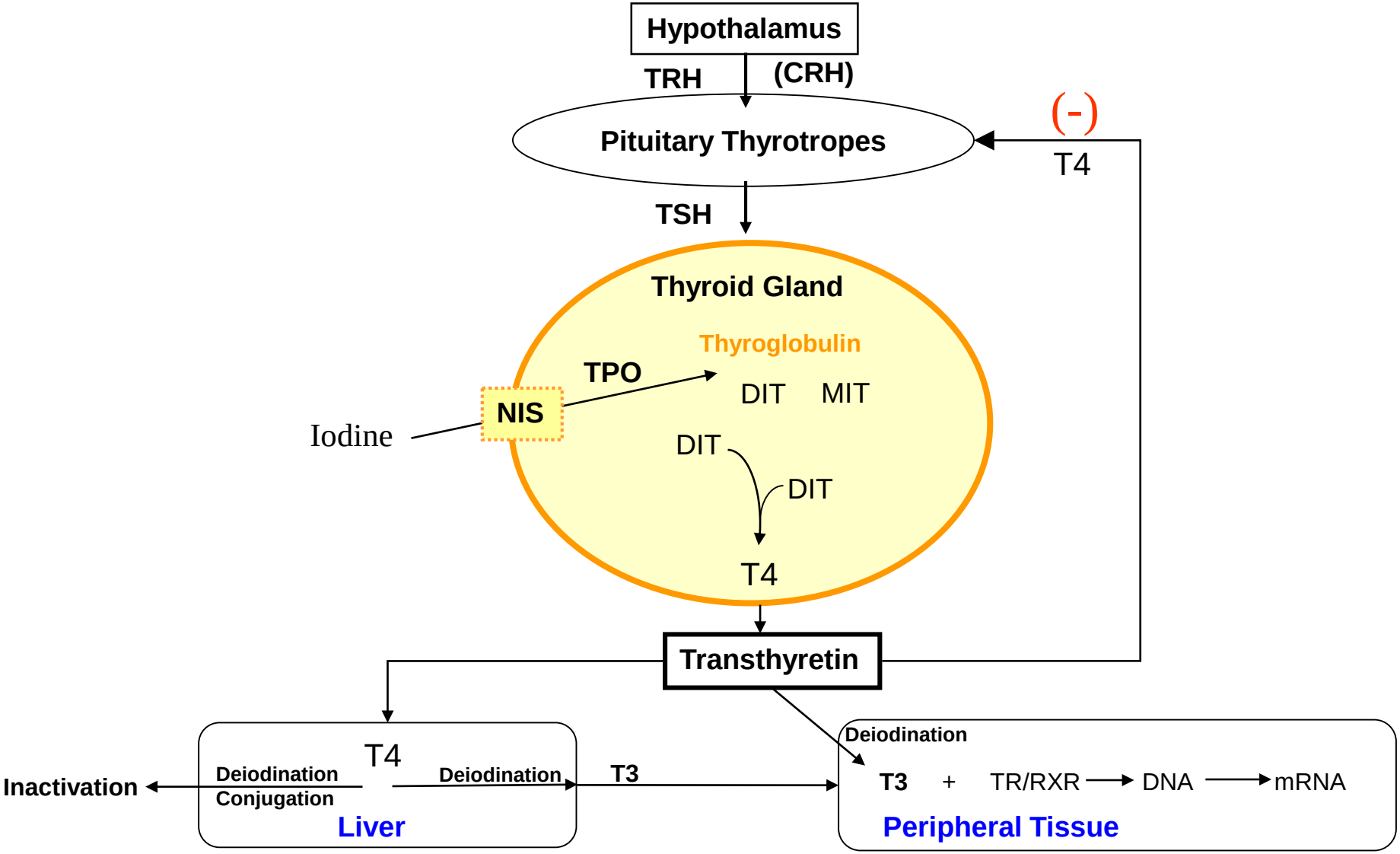
Thyroid Follicular Cell



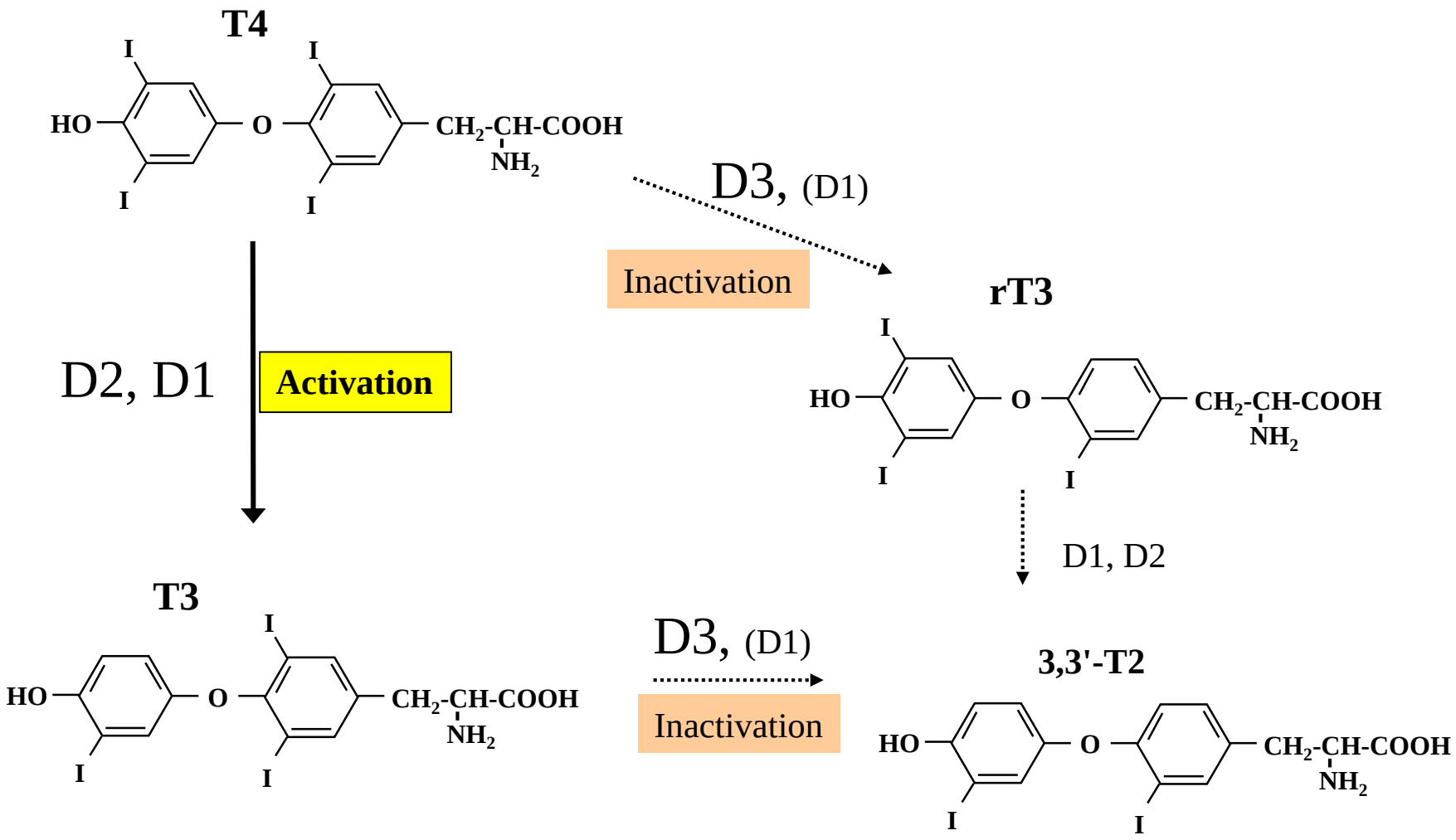




Thyroid Hormone Regulation



Deiodinase Activities



Rationale for a Frog Metamorphosis Model

- Metamorphosis is mediated by thyroid hormone
  - Resulting in great sensitivity to disruption
  - Easy apical observations
- Molecular events are well characterized
- Easy to raise and test in the laboratory
  - *Xenopus laevis*
  - *Xenopus tropicalis*

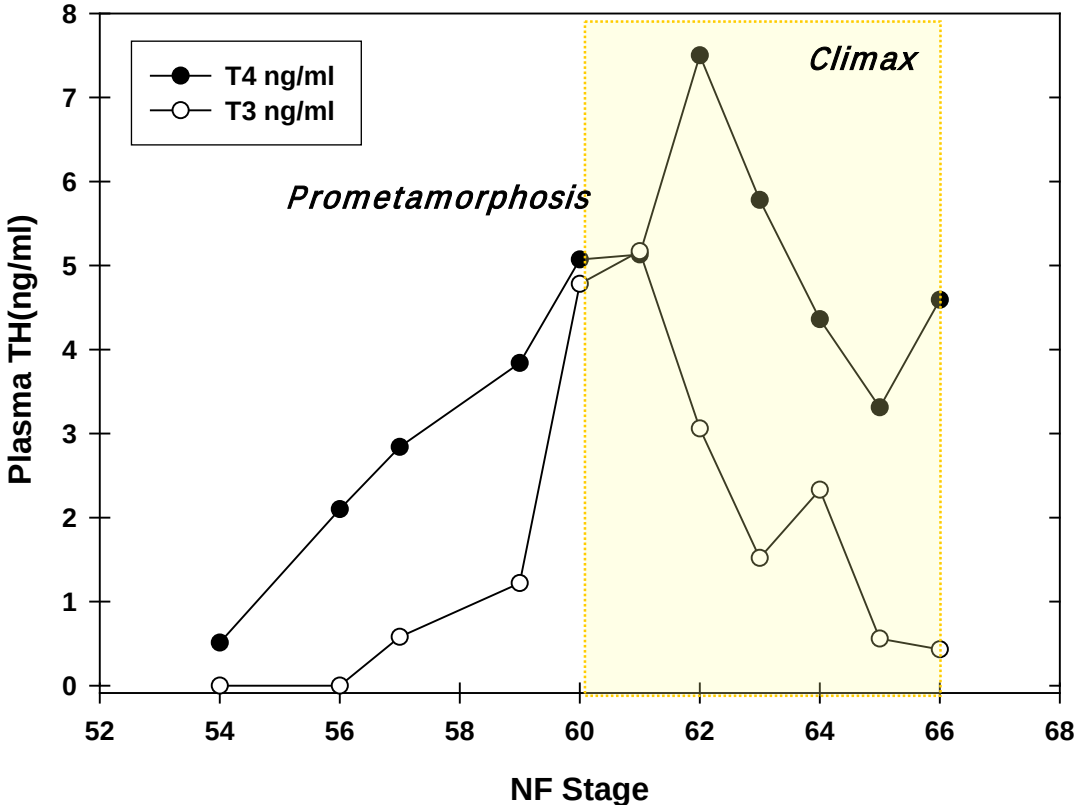
EDSTAC Tier 1 Frog Metamorphosis Assay

- African Clawed Frog, *Xenopus laevis*
- Expose at Stage 60
  - Coincident with endogenous TH peak
- 14 Day Exposure Through Stage 66
- Tail Length
- Resorption faster than controls = agonist
- Resorption slower than controls = antagonist

Xenopus Metamorphosis

X. laevis Plasma Thyroid Hormones

Leluop and Buscaglia 1977



Analysis of EDSTAC- Proposed Amphibian Metamorphosis Assay

EDSTAC-Proposed Amphibian Metamorphosis Assay was not ready for use as a screening tool

1) Developmental stage 60 is relatively insensitive
2) Tail tissue is relatively insensitive
3) Changes in tail resorption rates not diagnostic
4) Insufficient data on known agonists/antagonists
5) Interaction with other endocrine systems is source of uncertainty

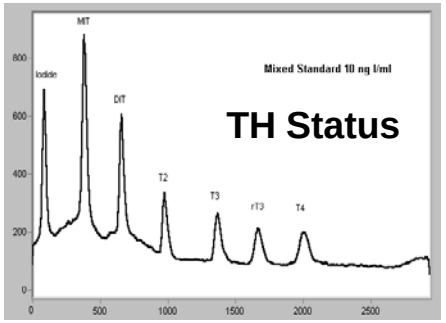
MED Thyroid Project Objectives

- Develop a whole organism based screening assay for the agency (determine appropriate stage)
- Conduct studies with known HPT disruptors
- Develop the appropriate diagnostic measure to establish confidence
  - What are the appropriate tissue level endpoints?
  - Can gene and protein expression be used as indicators of thyroid axis disruption?

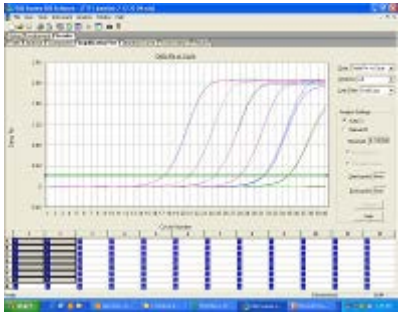
Research Approach

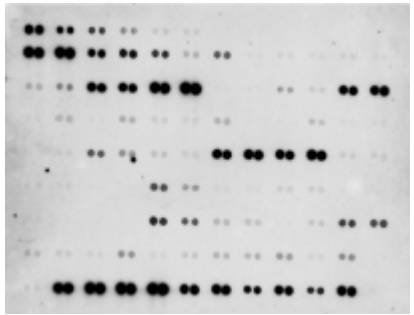
Chemical Exposure

Organismal Response

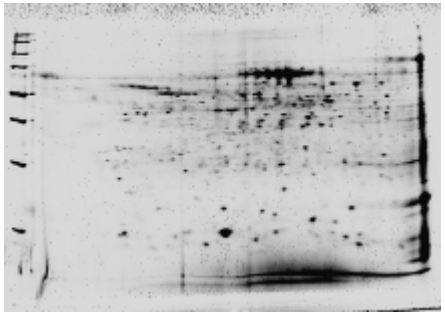


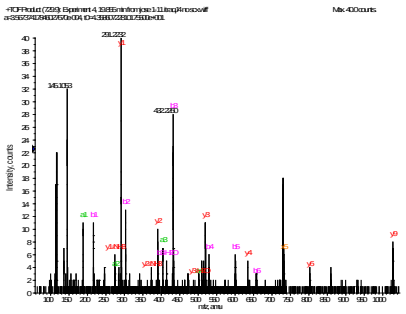
Gene Expression



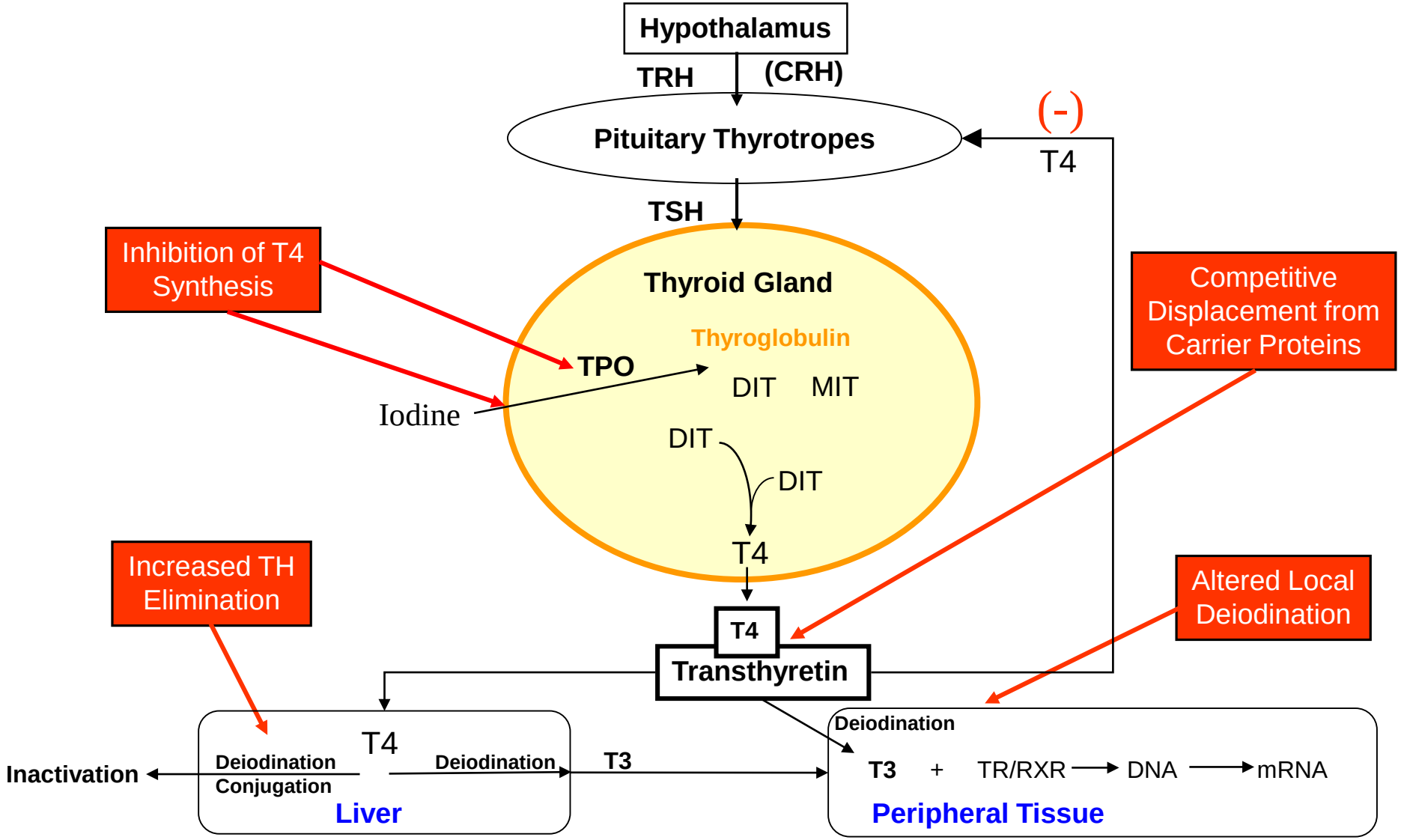


Protein Profiling





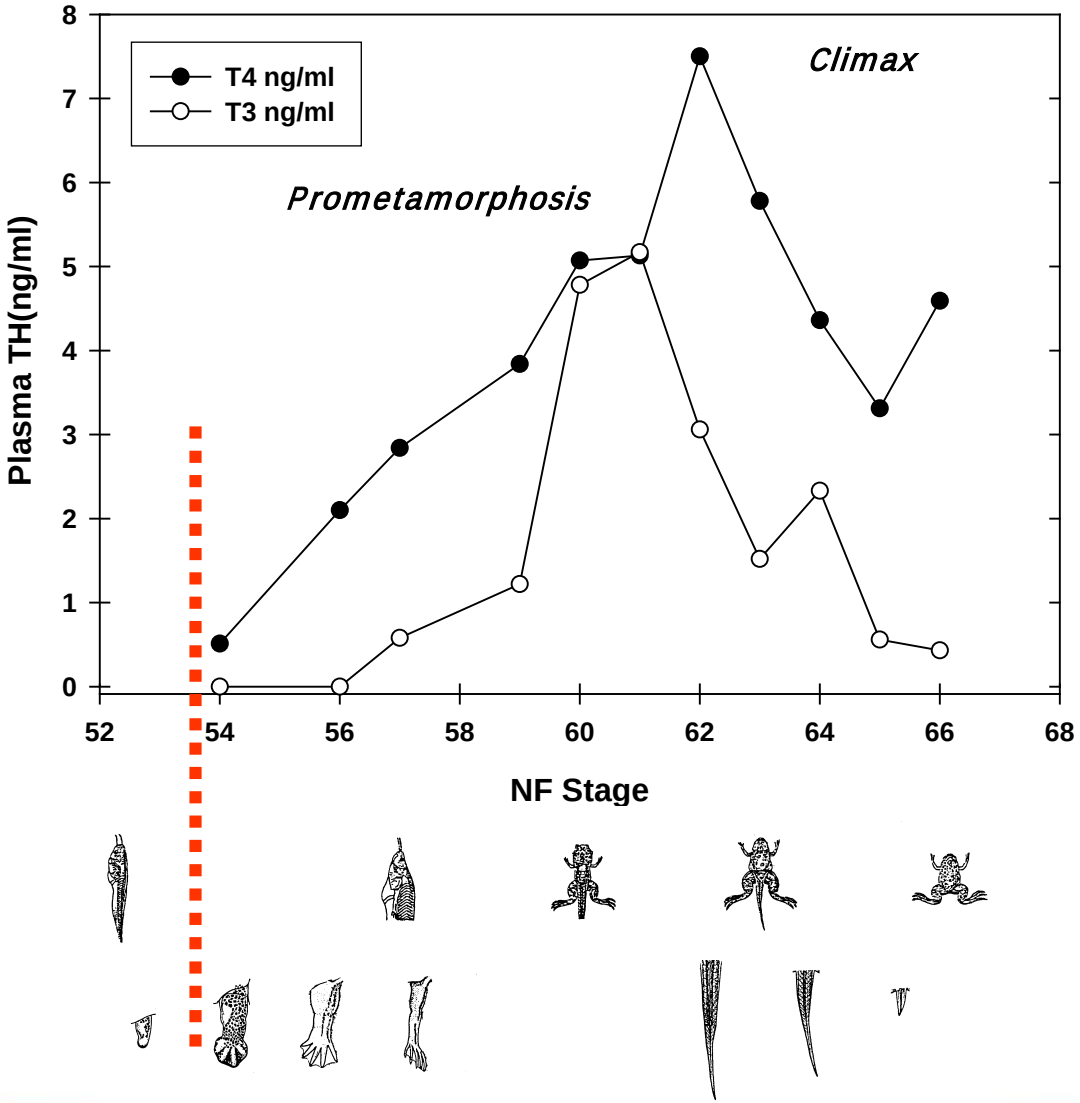
Thyroid Disruption Pathways



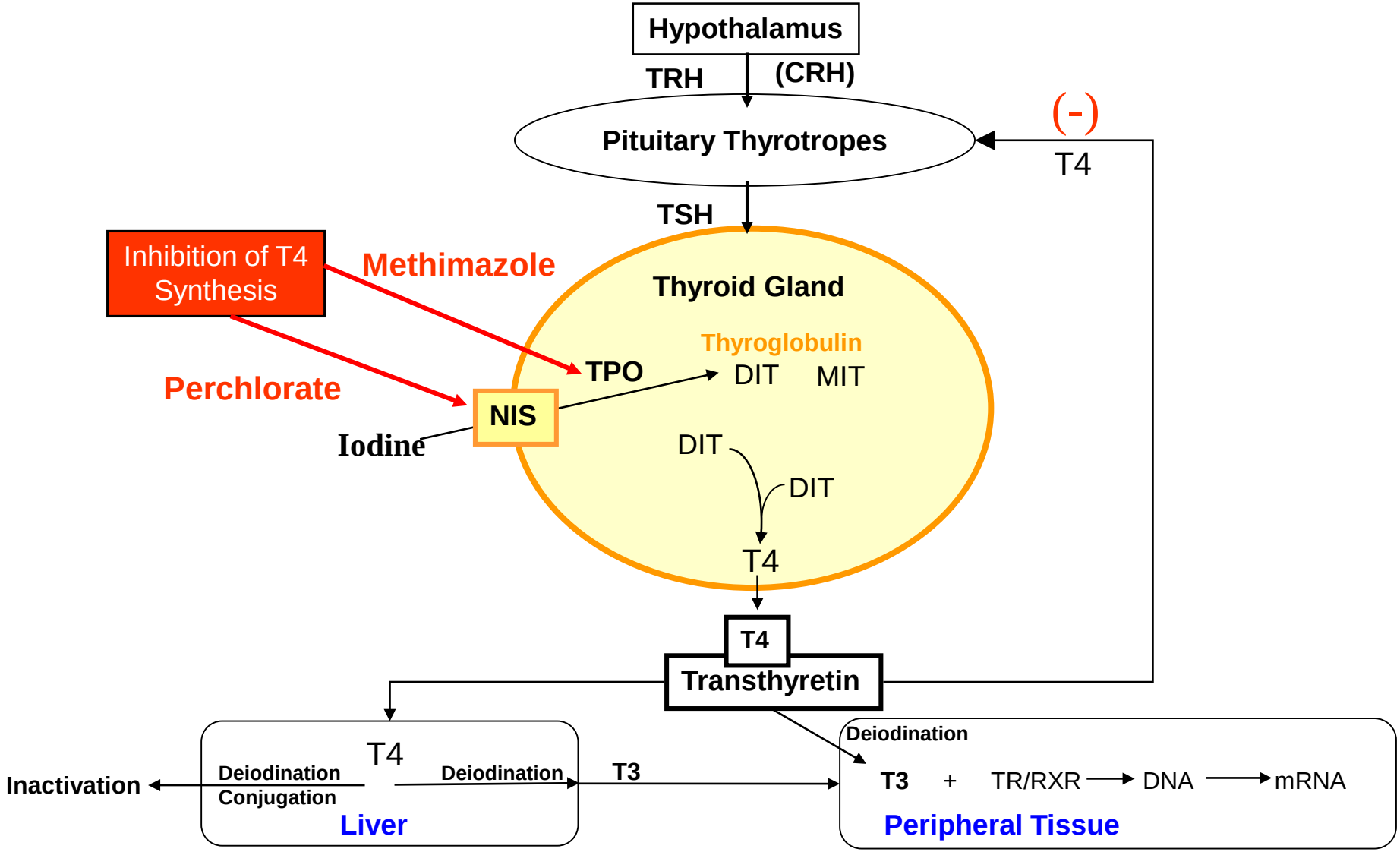
Experimental Approach: Organismal Response

X. laevis Plasma Thyroid Hormones

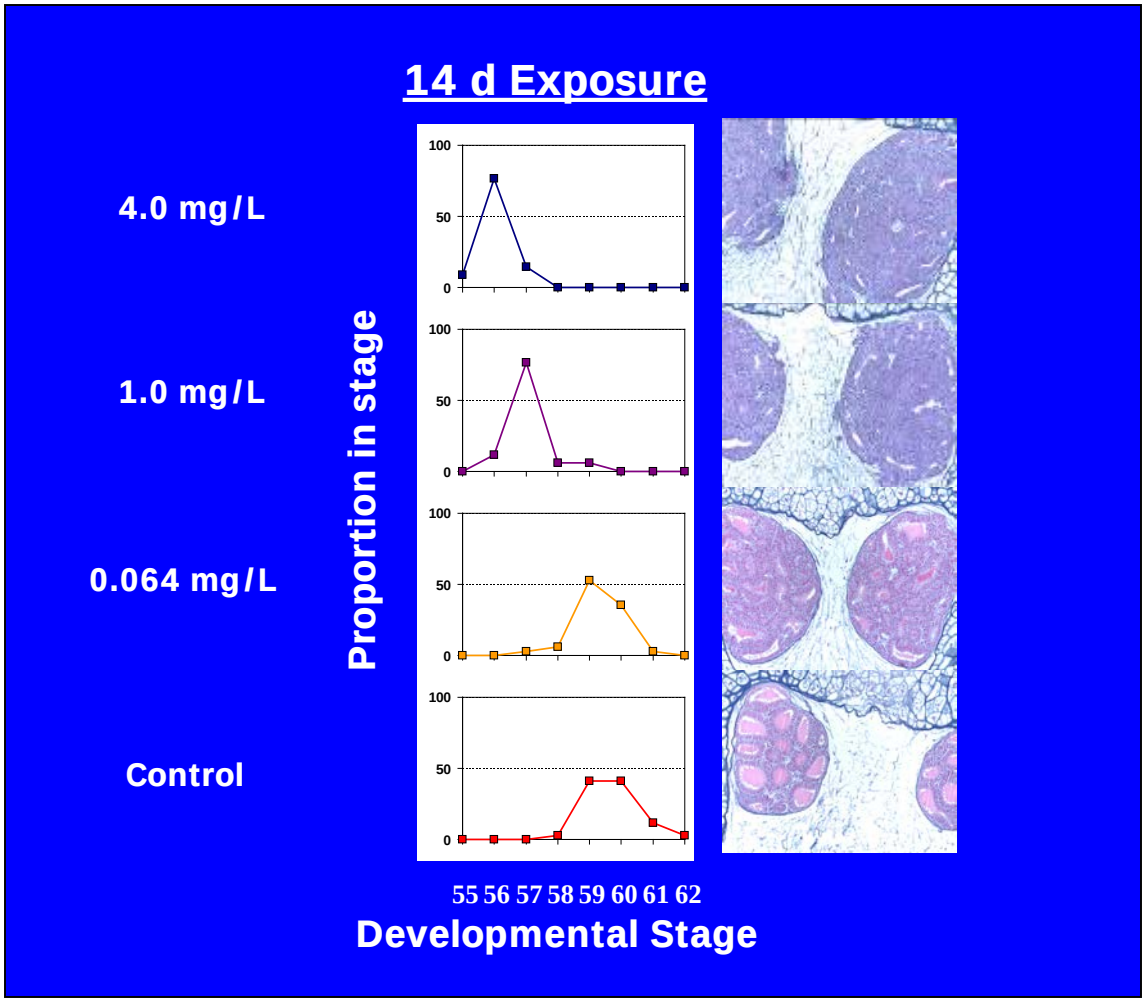
Leluop and Buscaglia 1977



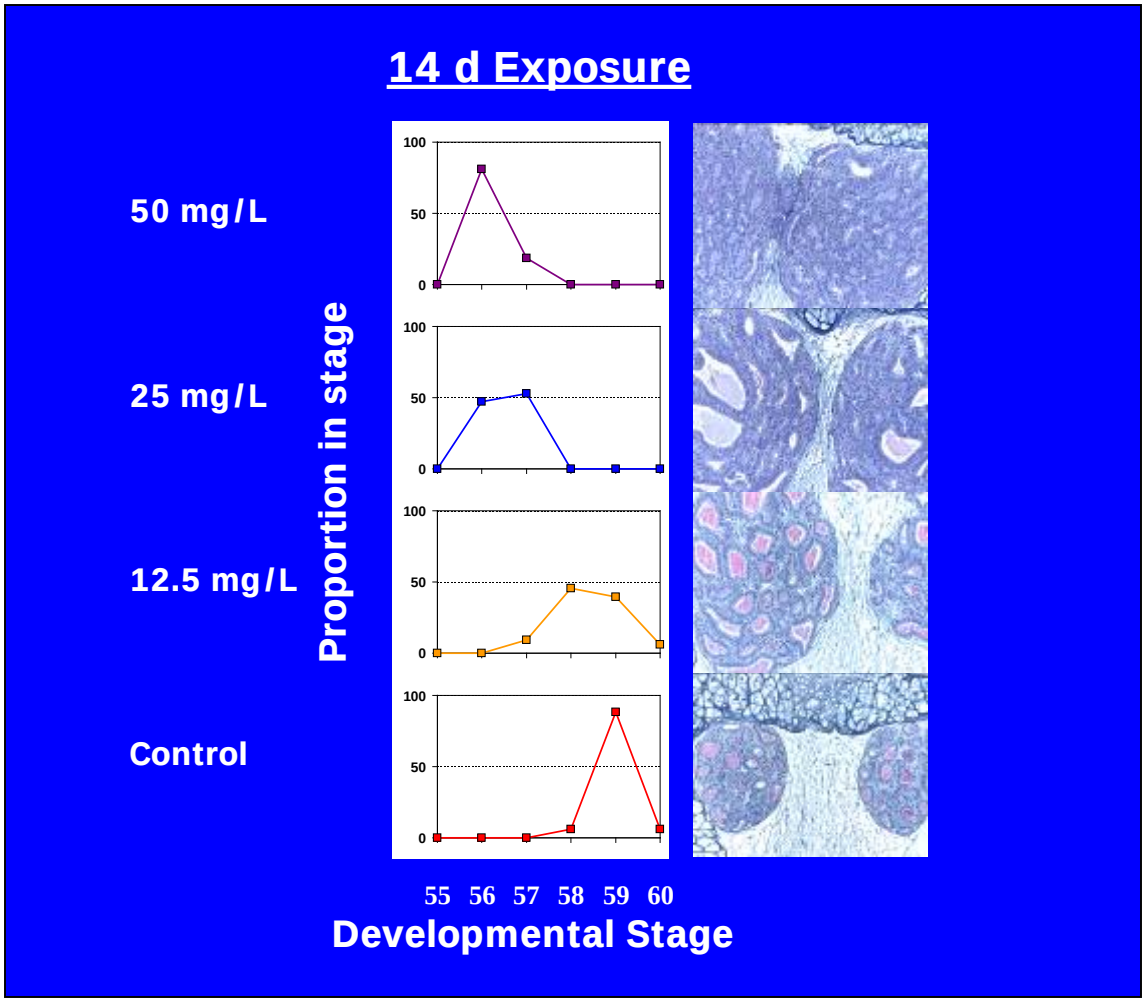
Thyroid Disruption: T4 Synthesis



Effect of Perchlorate on Development and Thyroid Histology



Effect of Methimazole on Development and Thyroid Histology



Summary of Findings

- *X. laevis* is sensitive to model thyroid pathway modulators
  - Methimazole, 6-PTU, Perchlorate
- Stage 54 is as sensitive as stage 51
- Thyroid histology is an essential component of assay
  - More sensitive than developmental rate
  - Diagnostic
  - Rapid

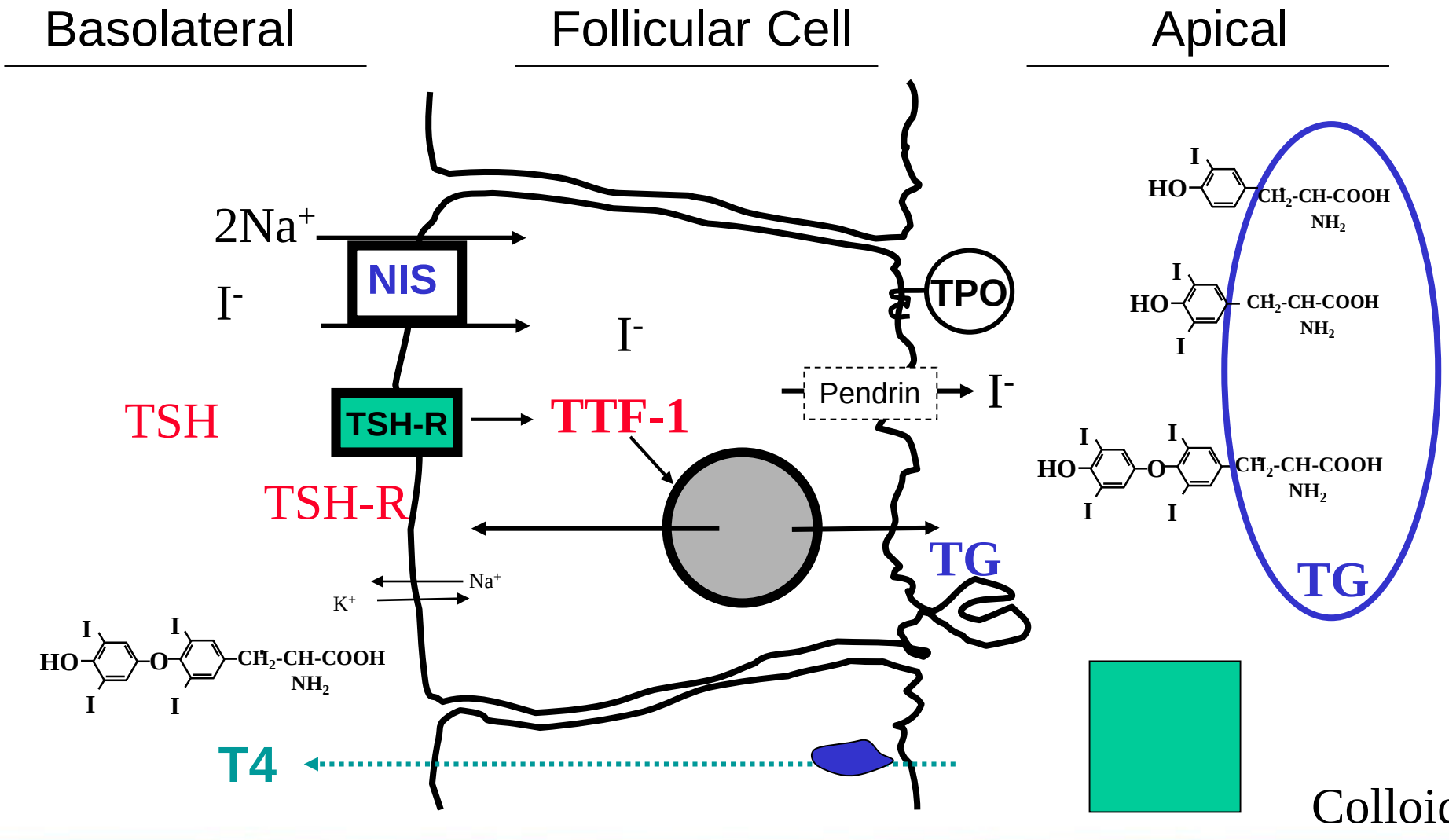
Develop a Diagnostic Research Approach

- Link chemical-biomolecule interaction to whole organism effects
- Develop computational approaches

Developing Diagnostic Indicators

- Look at things you know (QPCR, ELISA..)
  - Normal development
  - Chemical exposure
- Go Fishing
  - Gene arrays
  - 2D protein analysis
  - MS based protein analysis

Endpoints in the Thyroid Gland

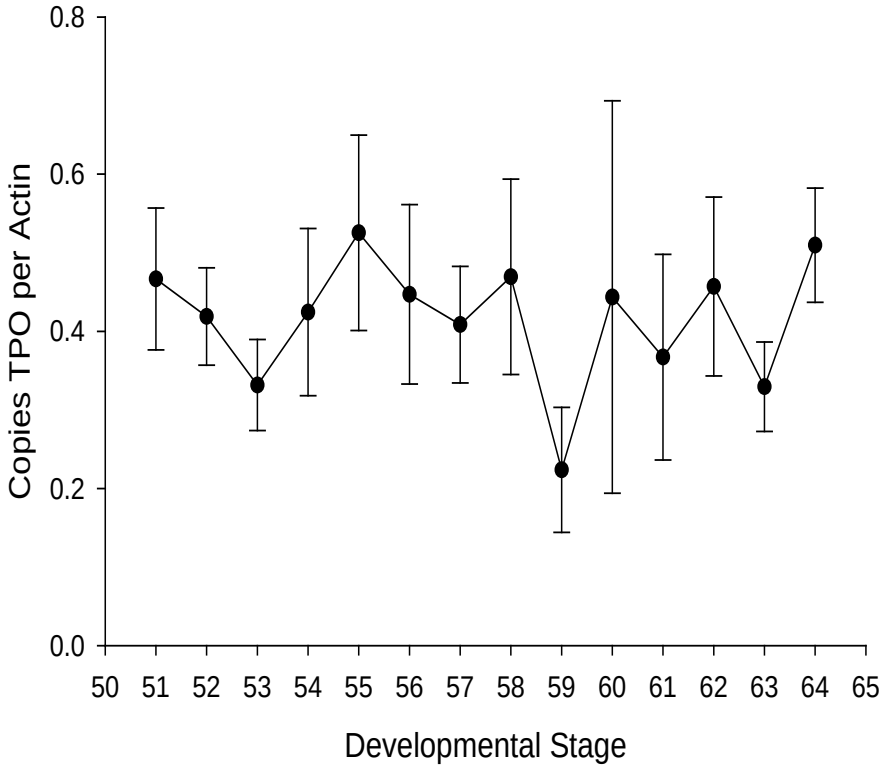


Approach

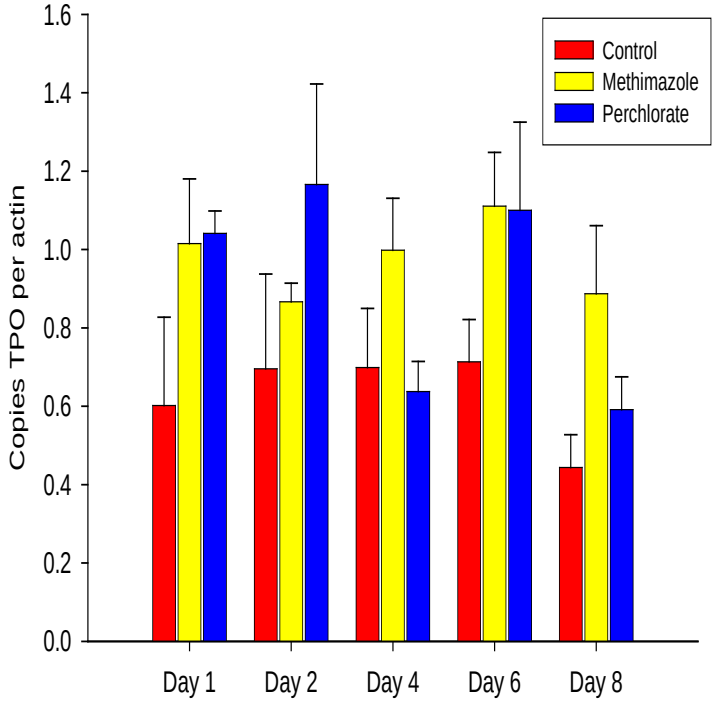
- Examine gene expression during normal metamorphosis
- Examine gene expression following in vivo exposure
- T4 production and gene expression by thyroid explant cultures
- Gene array analysis following in vivo exposure

Gene Expression in Thyroid Gland Thyroid Peroxidase

Developmental Expression

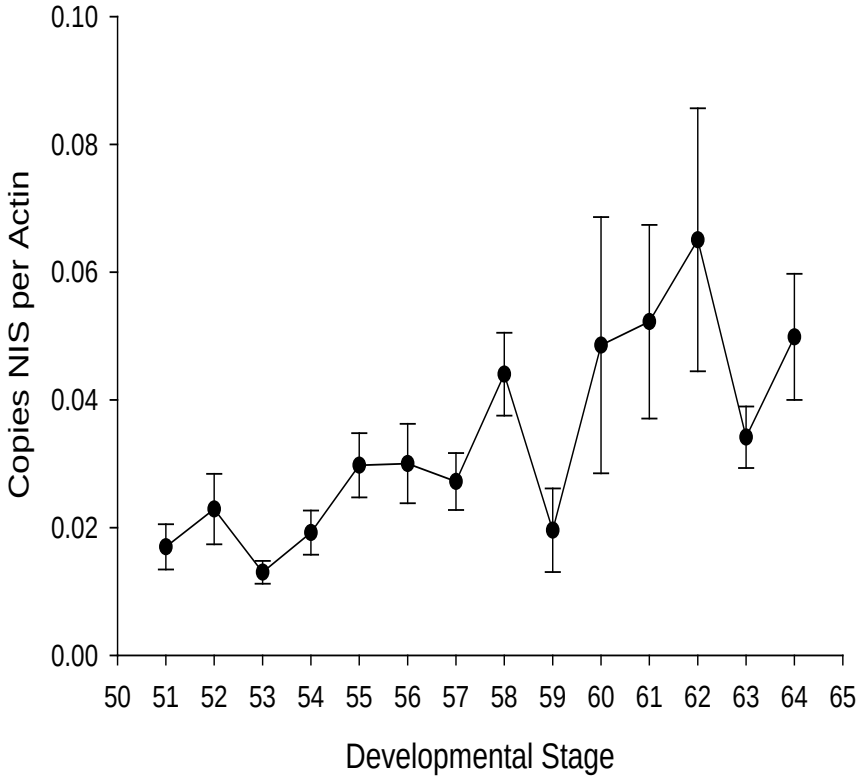


Chemical Exposure

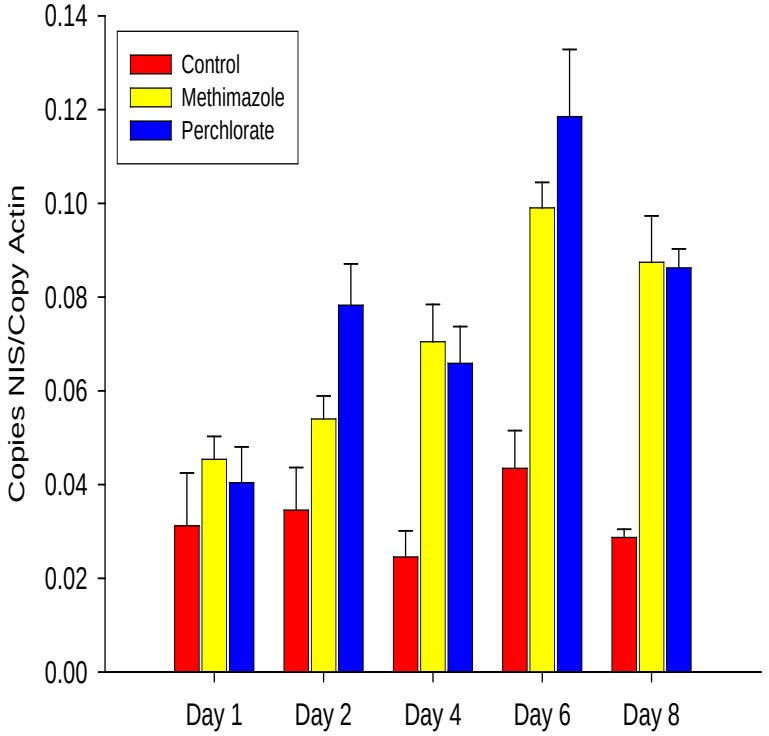


Gene Expression in Thyroid Gland Sodium/Iodide Symporter

Developmental Expression

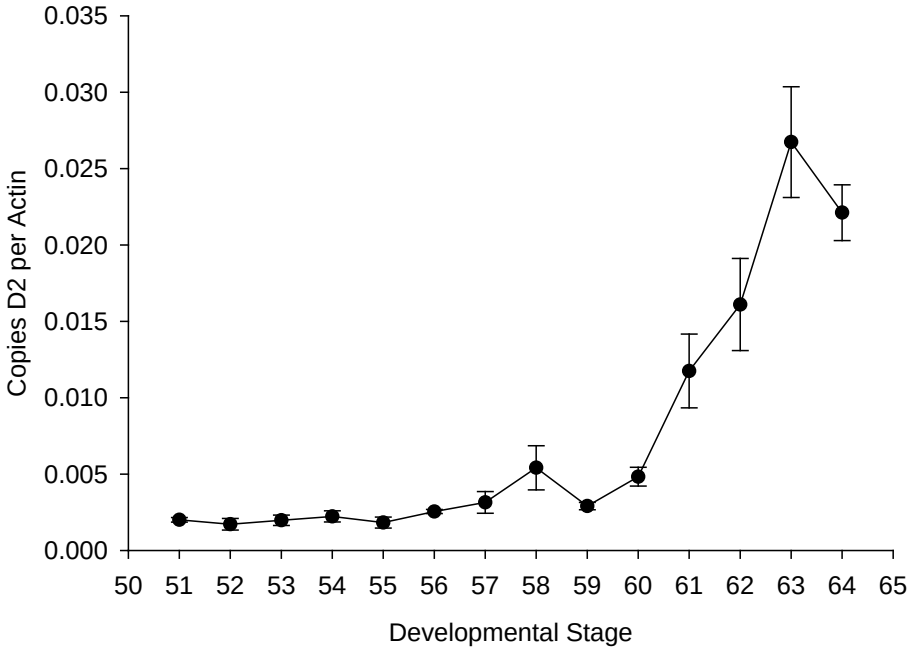


Chemical Exposure

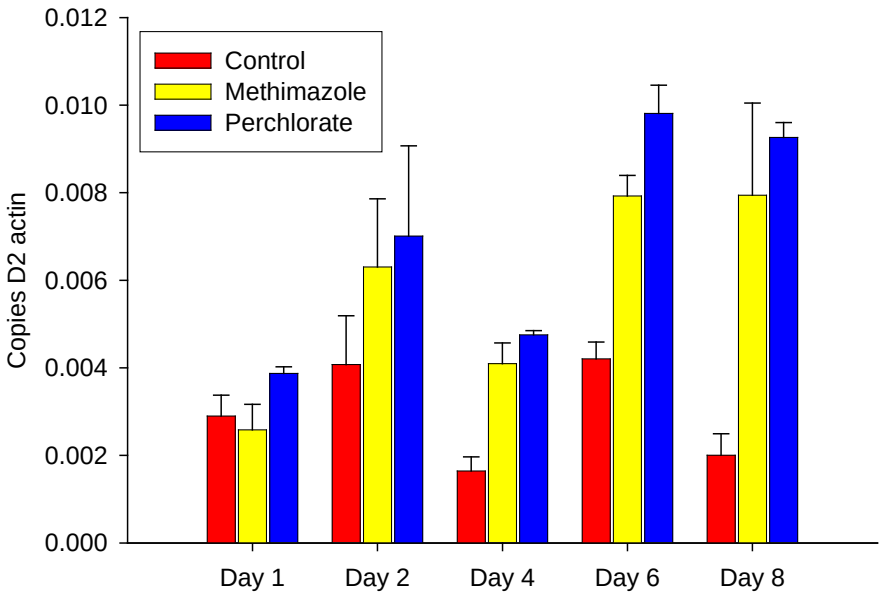


Gene Expression in Thyroid Gland
Type II Deiodinase

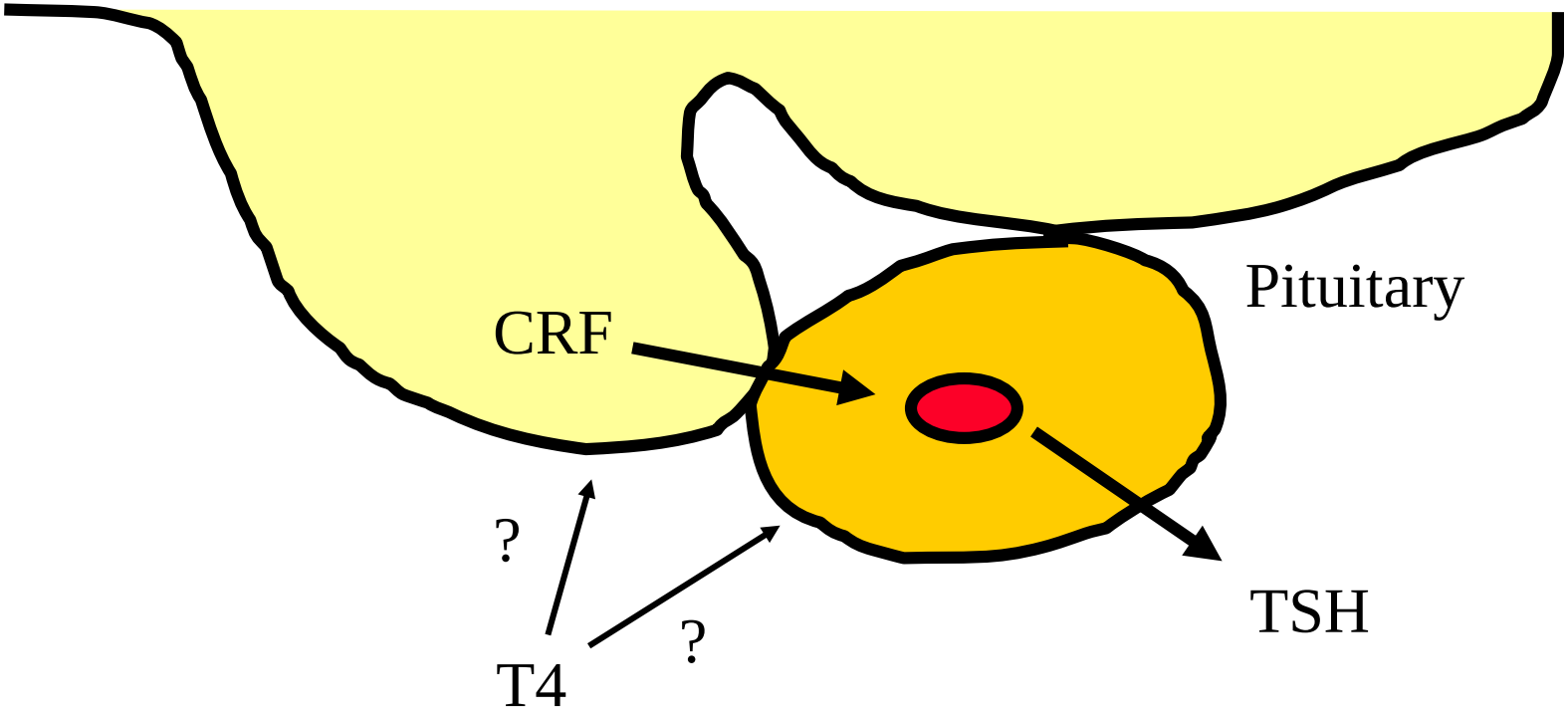
Developmental Expression



Chemical Exposure

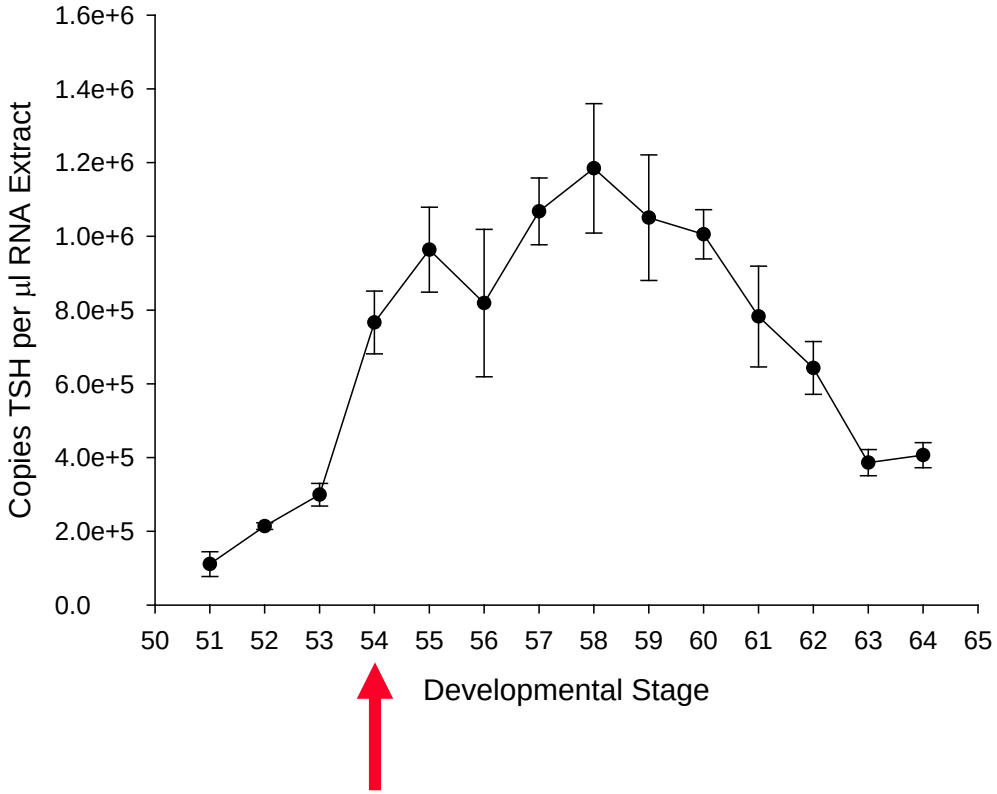


What could we look at in the pituitary?

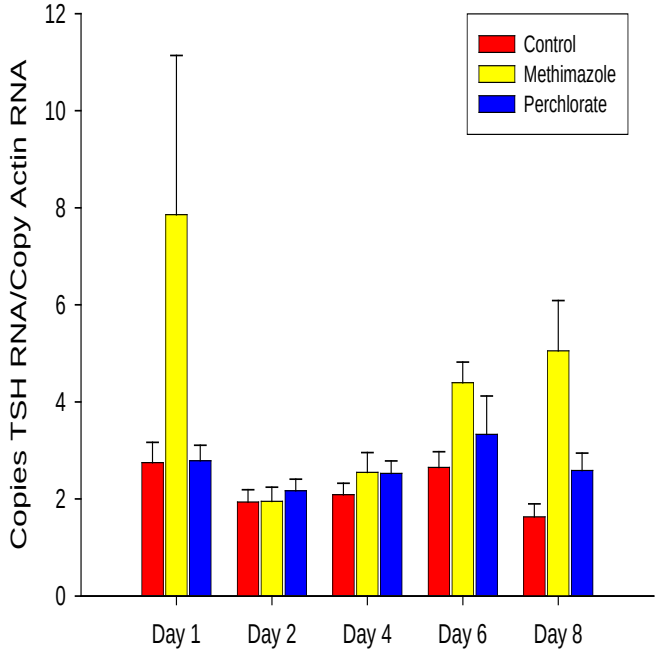


Gene Expression in Pituitary Thyroid Stimulating Hormone

Developmental Expression

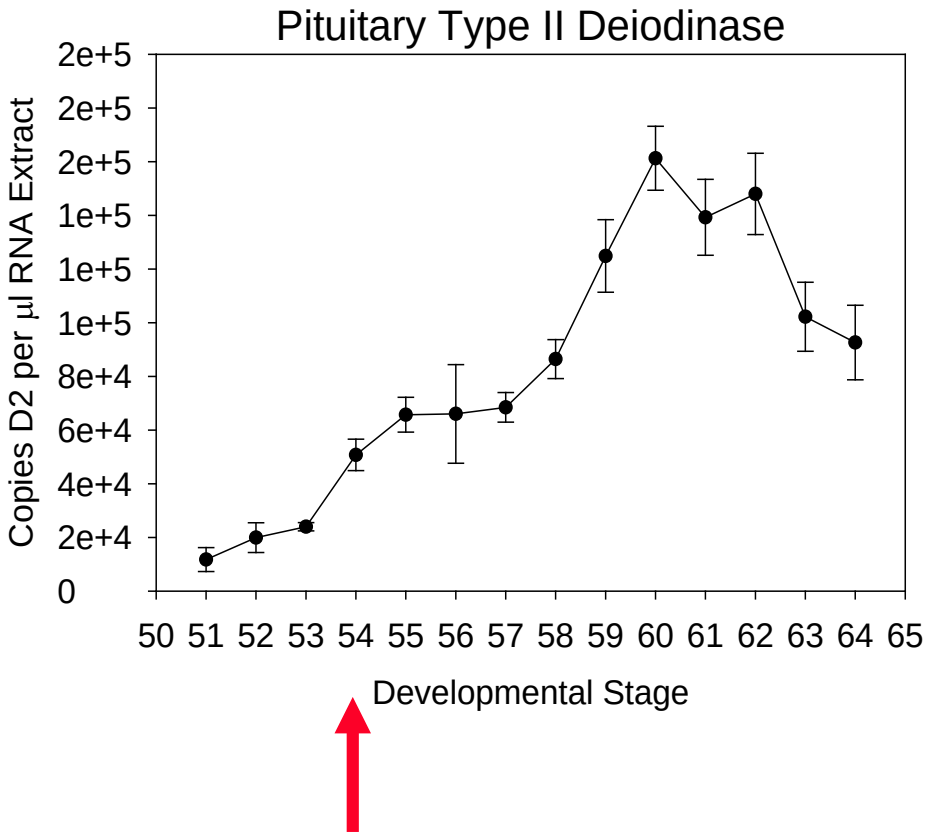


Chemical Exposure

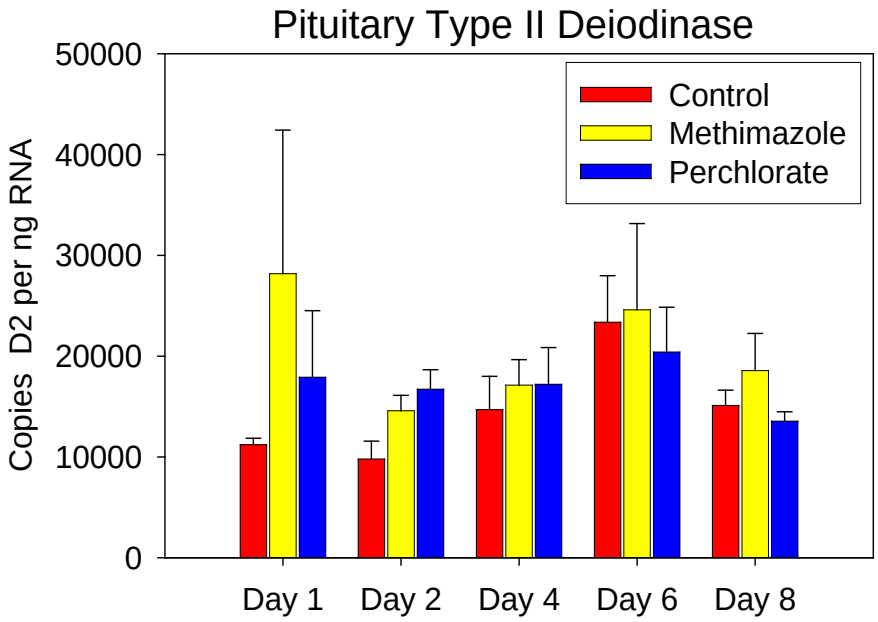


Gene Expression in Pituitary Type II Deiodinase

Developmental Expression



Chemical Exposure



Research Approach

In vitro models: tissue explants

- Thyroid
- Pituitary
- Hypothalamus
- Co-cultures

Thyroid Gland Explant Culture

Dissect thyroid glands from pro-metamorphic tadpoles

Culture at 21°C in L-15 media

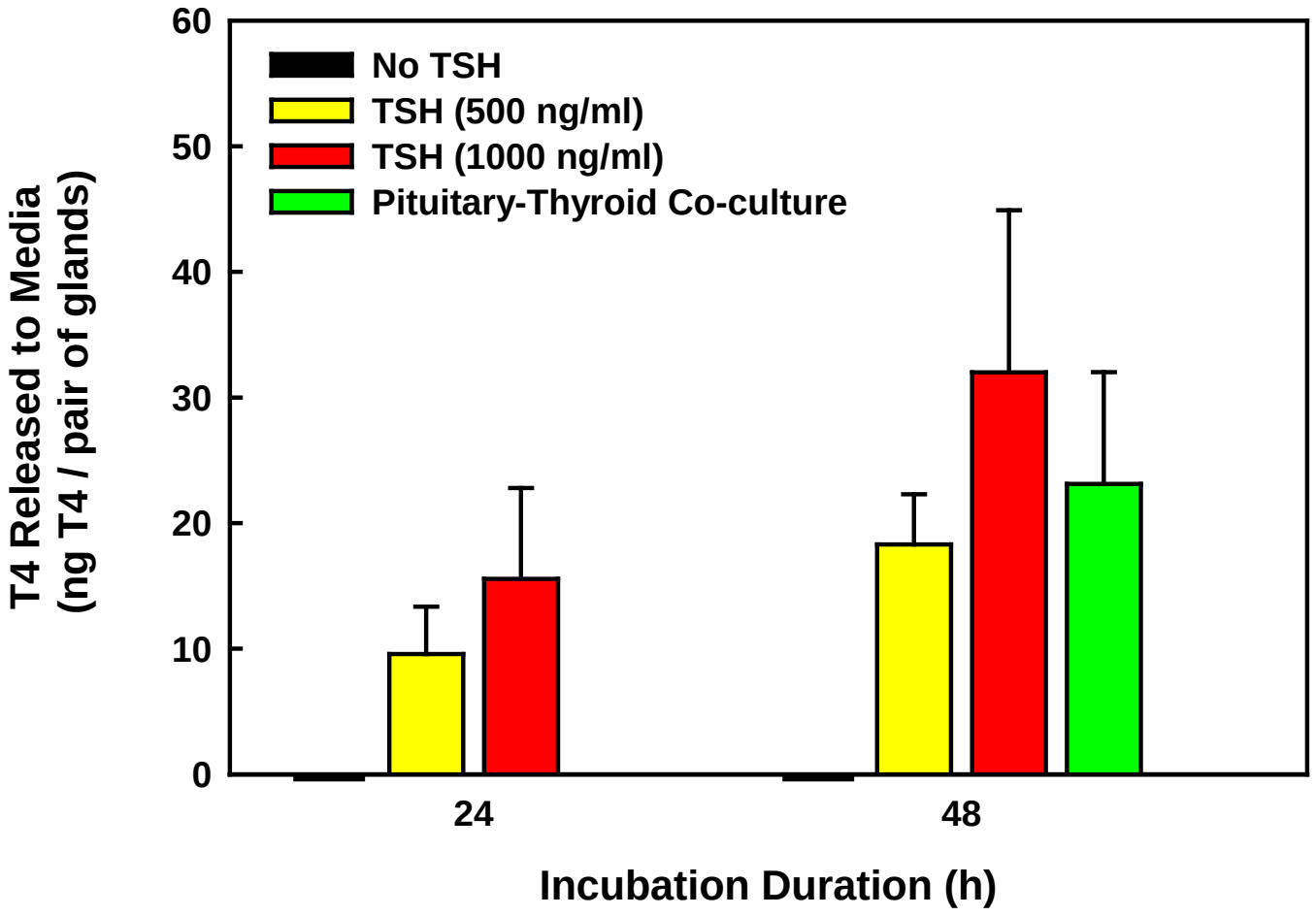
- 0.1% BSA
- potassium iodide
- antibiotic/antimycotic

Add bovine TSH to stimulate T4 production & release into media

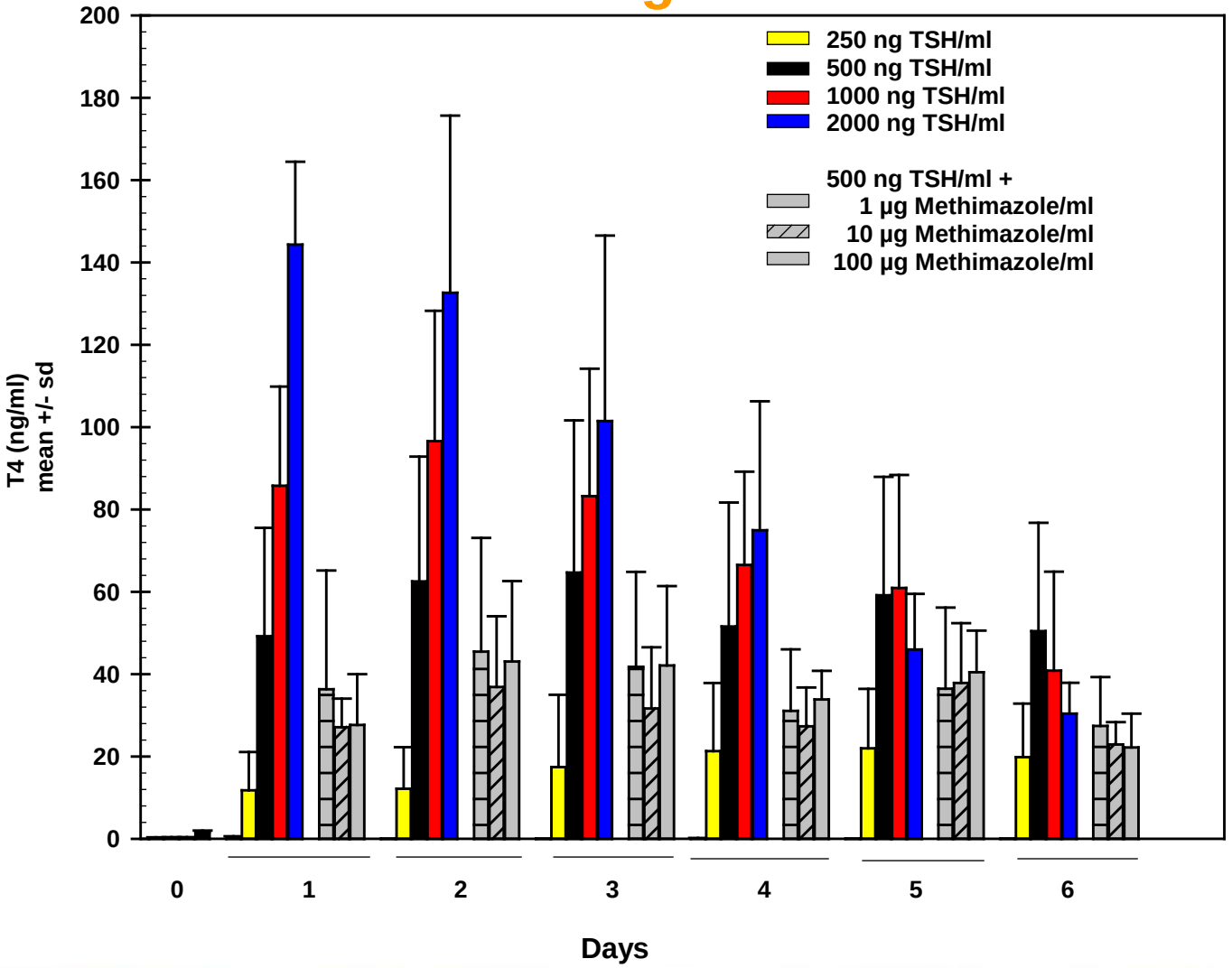
Collect media (24-144h) and determine T4 by ELISA

Collect glands for protein analysis or gene expression

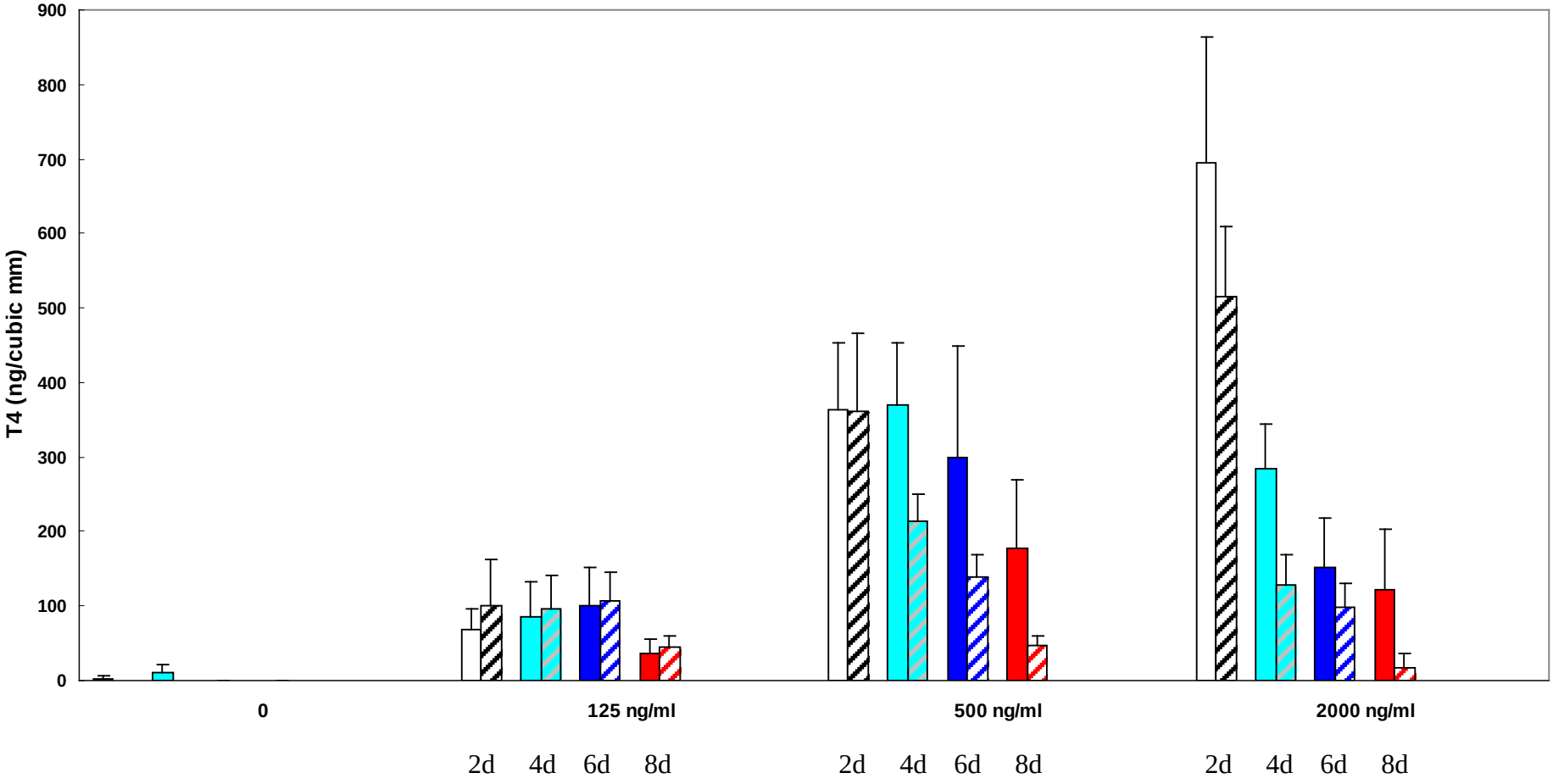
Thyroid Gland Culture
Effects of TSH on T4 release in culture



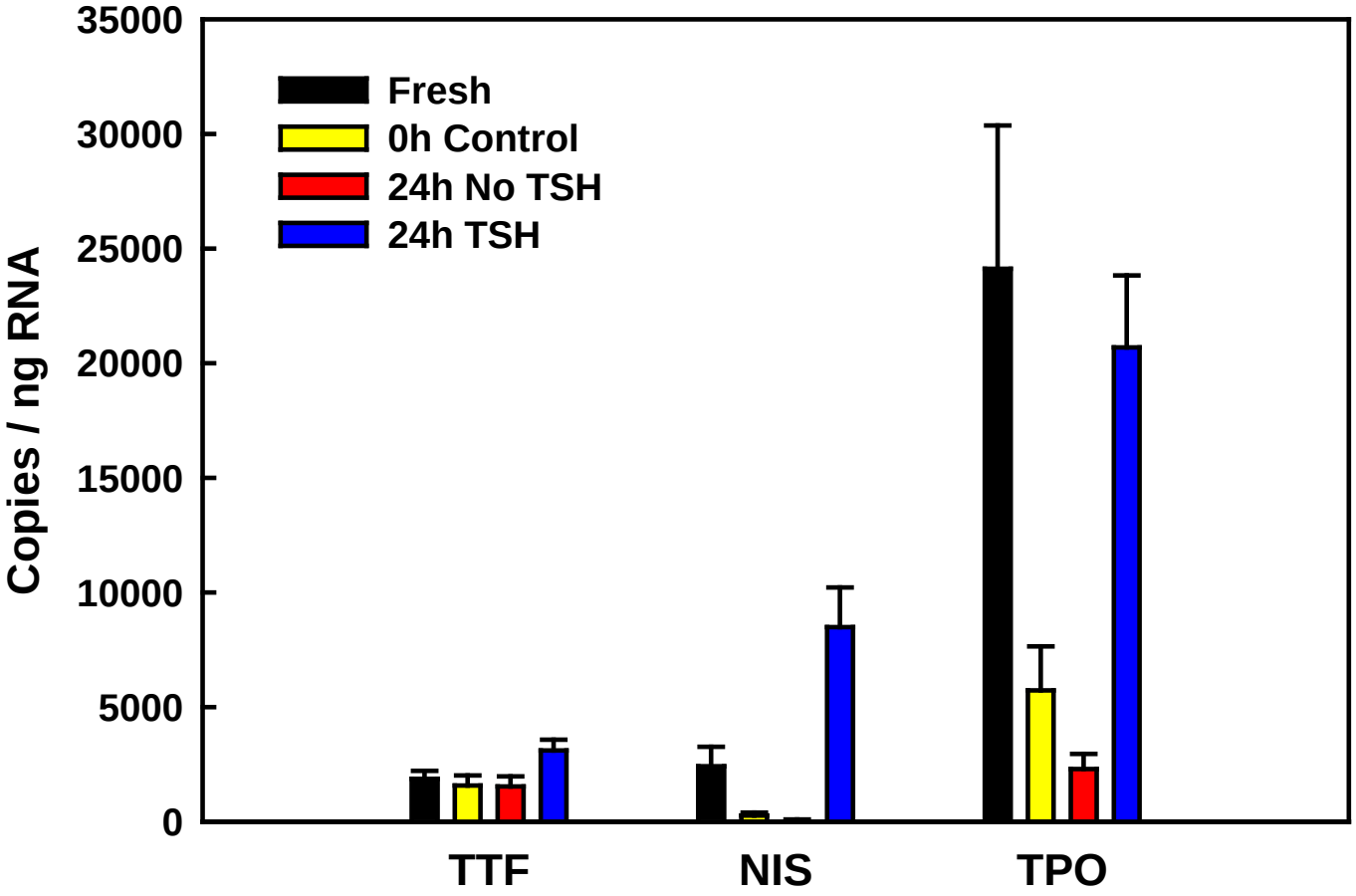
Thyroid gland culture
Response to TSH and methimazole
Stage 59



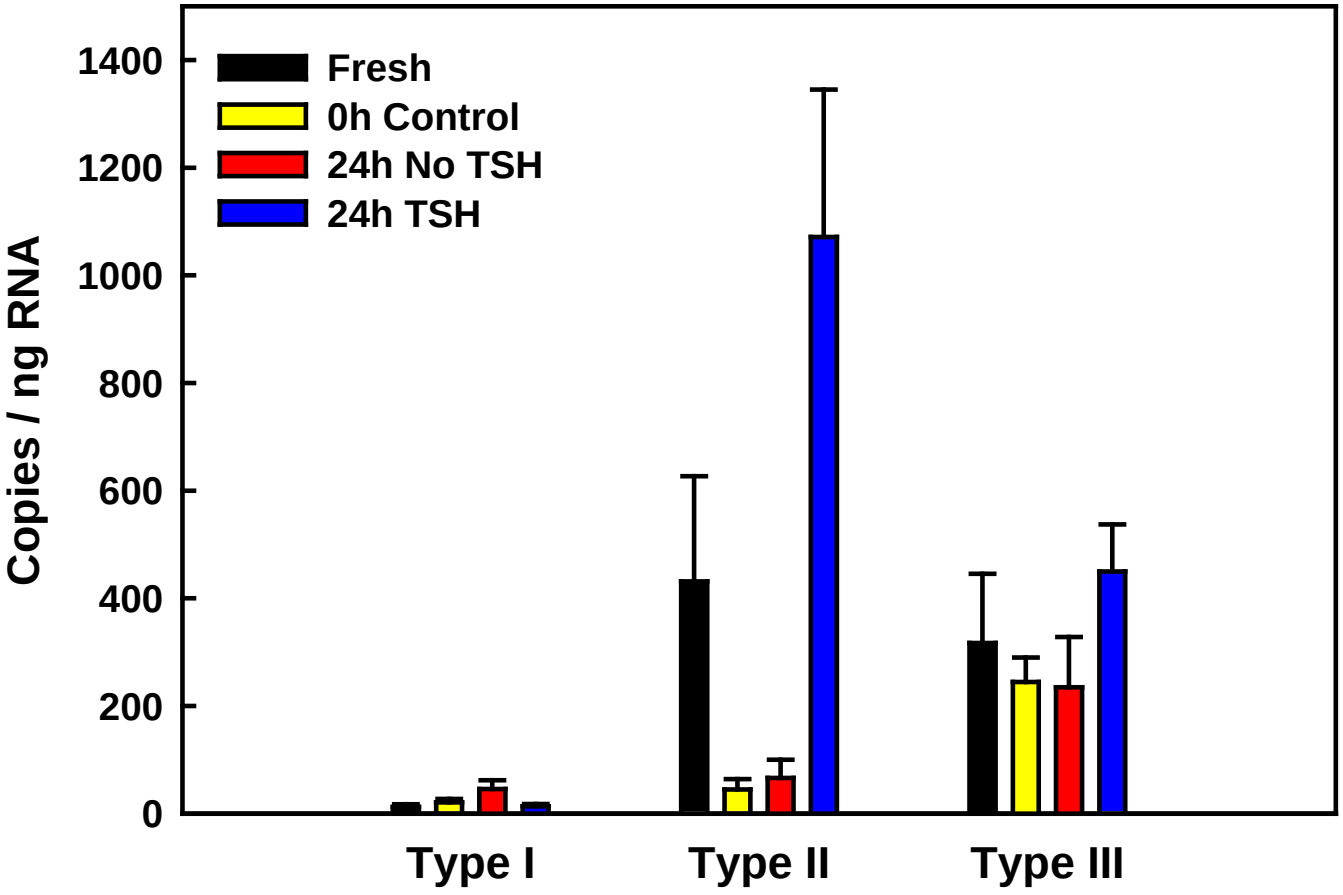
Thyroid gland culture
Response to TSH and methimazole
Stage 55



Thyroid Gland Culture: TSH regulation of T4 synthesis genes



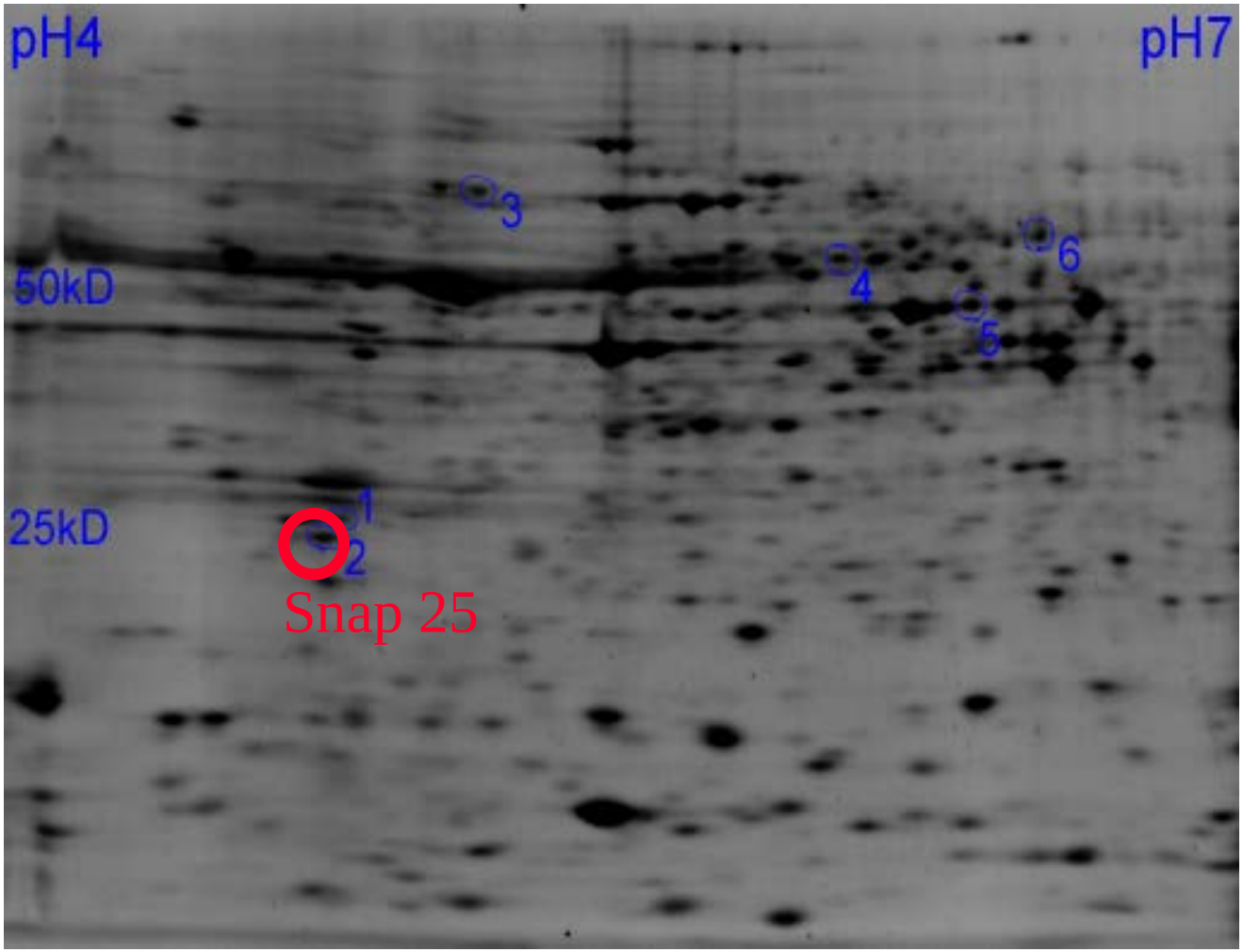
Thyroid Gland Culture
TSH Regulation of deiodinase in culture



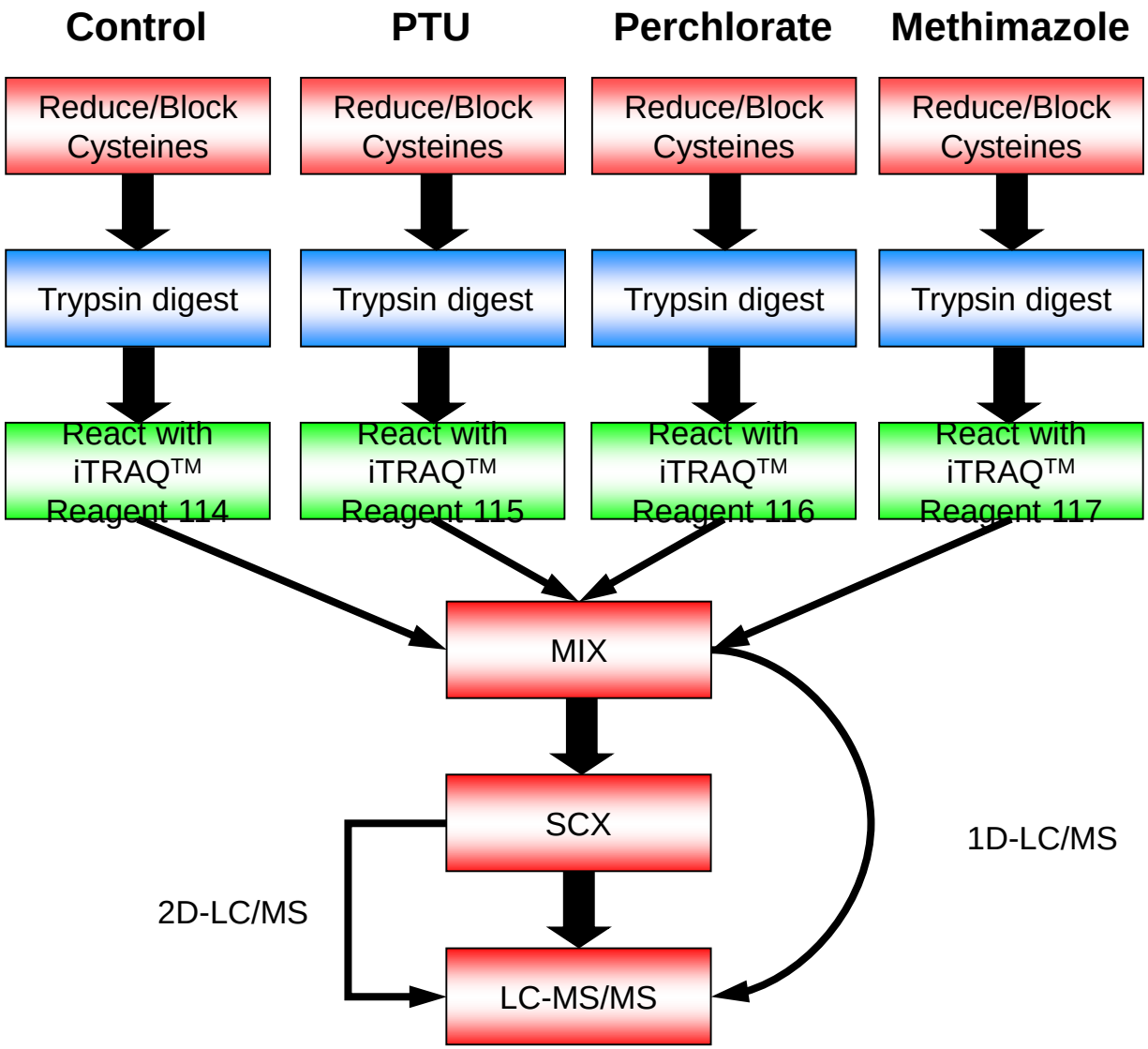
Protein Profiling

- Jose Serrano - MED
- Link protien changes with mode of action and whole organism outcome
- Treat with model thyroid axis inhibitors
  - Perchlorate (iodine uptake inhibitor)
  - PTU (TPO inhibitor)
  - Methimazole (TPO inhibitor)
- Remove Brain
- 2D gel and iTRAQ
- Characterize proteins changes common to two or more inhibitors

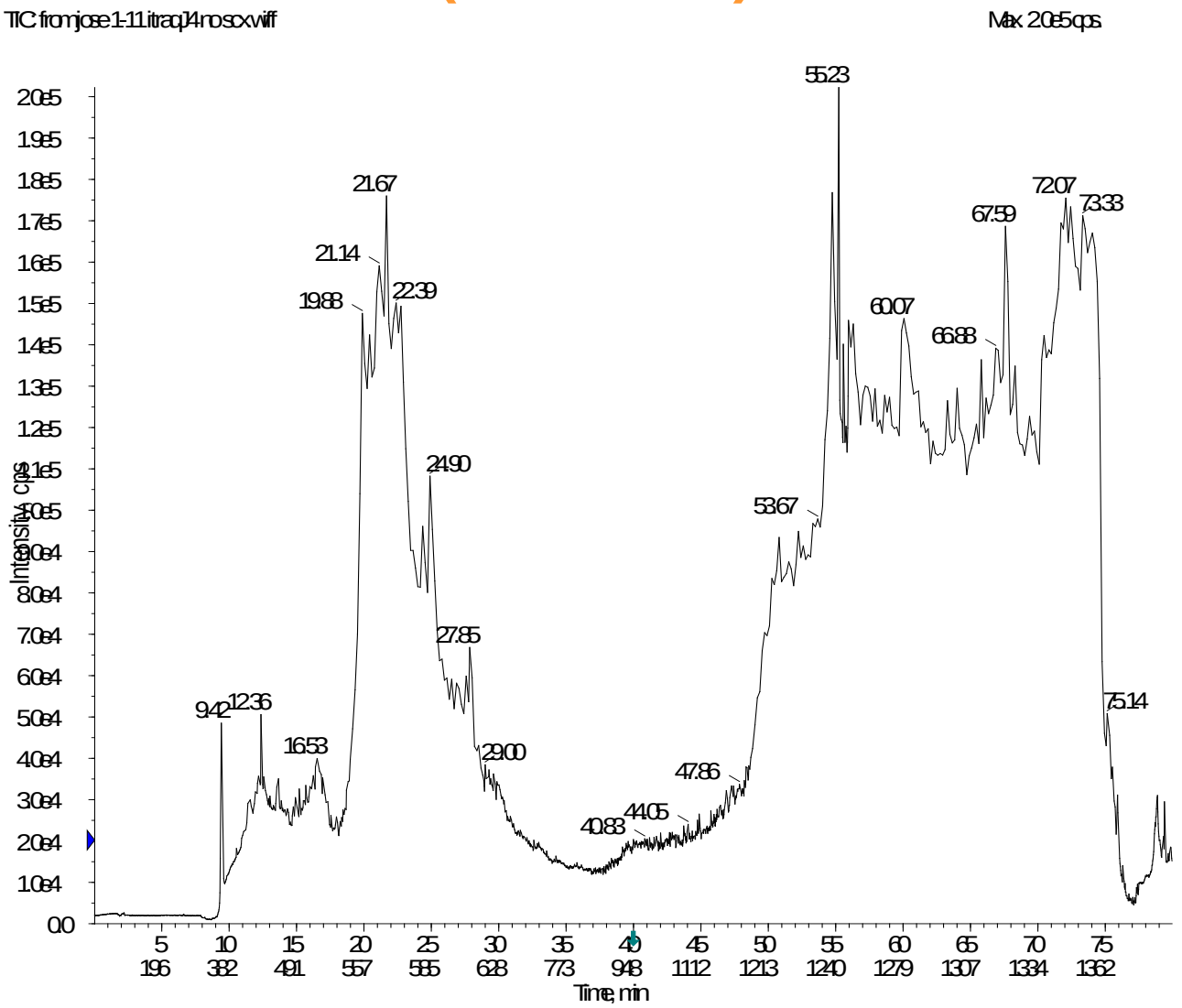
Gel of Brain Proteins From Perchlorate Treated Tadpoles

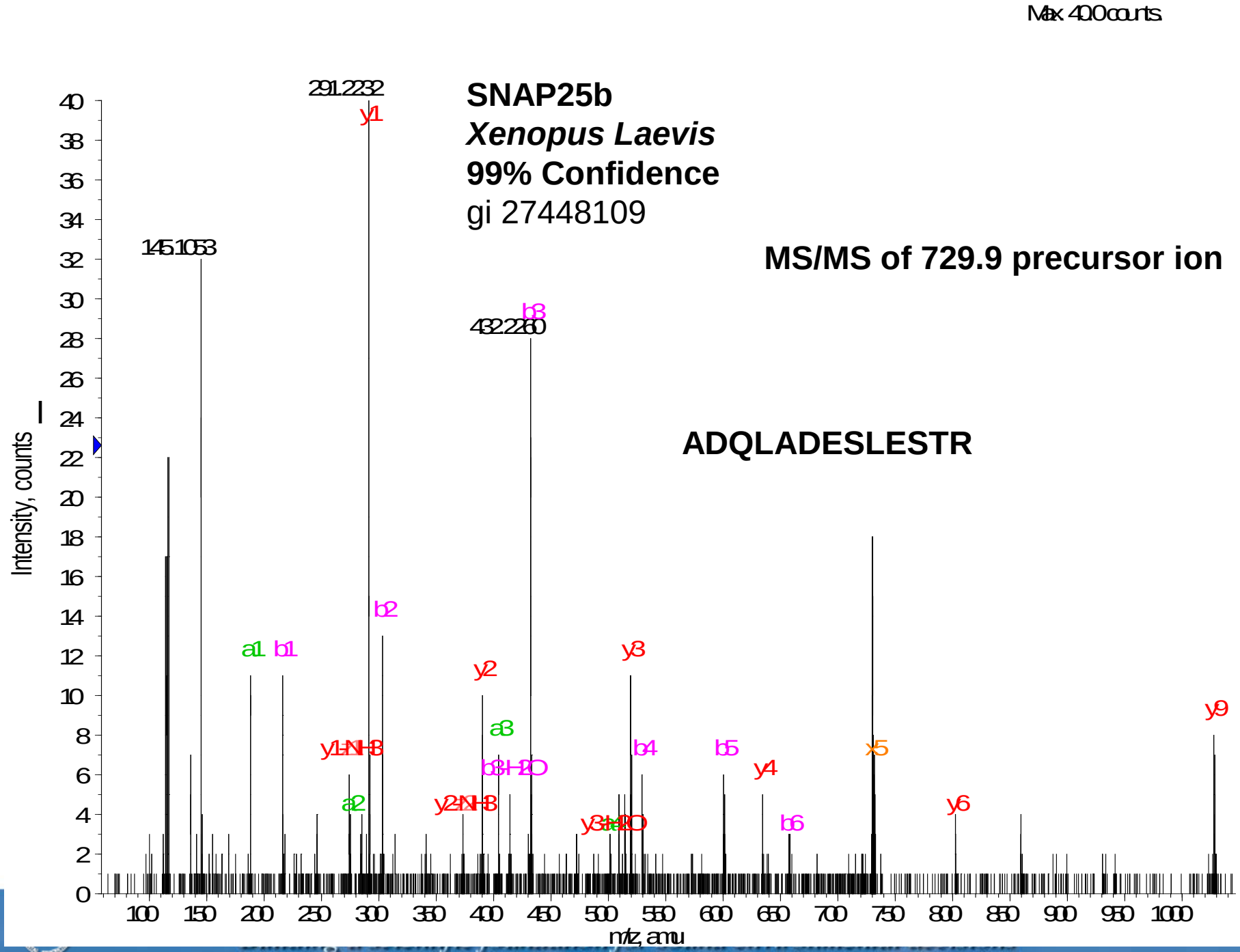


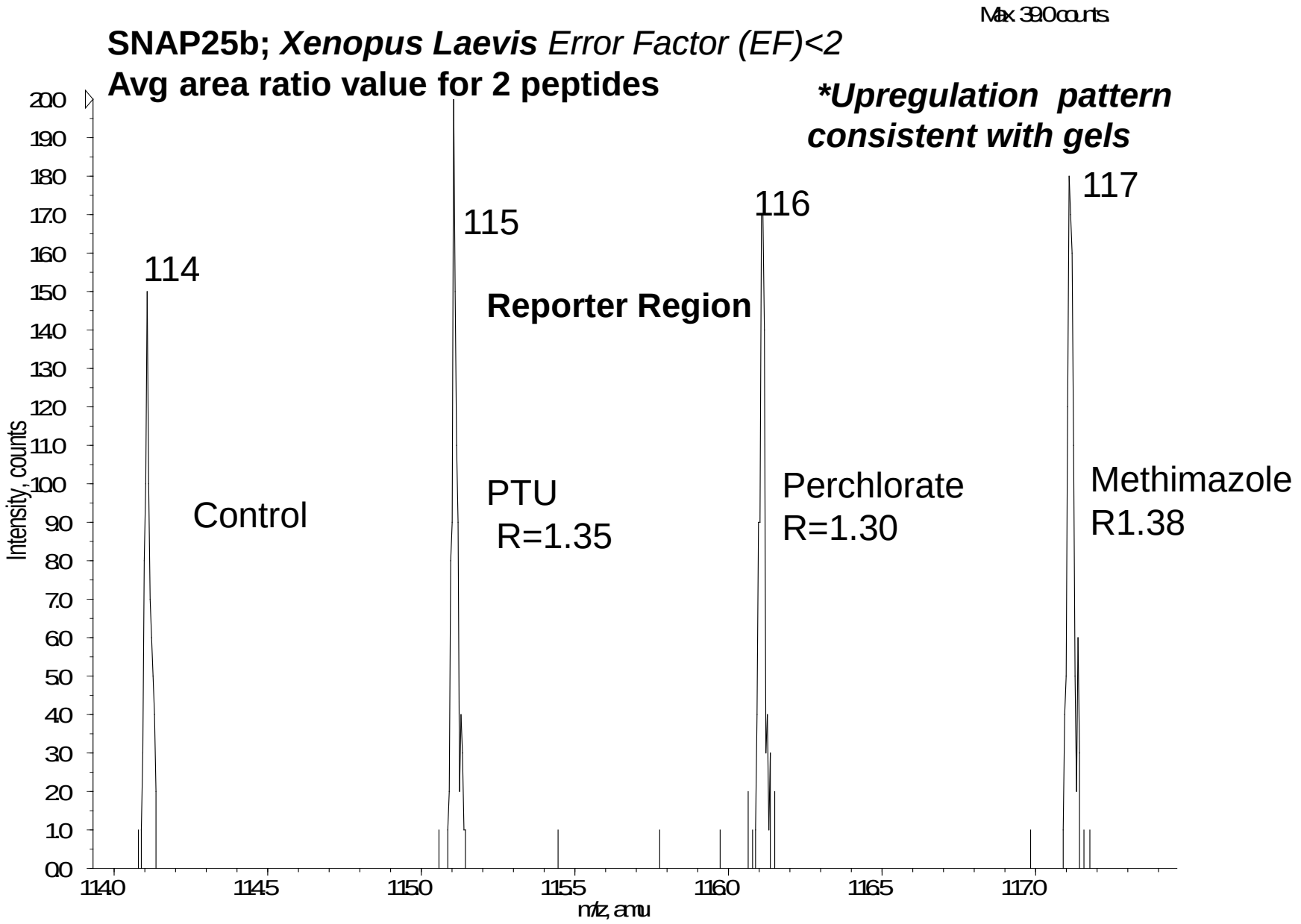
Methodology For iTRAQ Labeling and MS Analysis



Total Ion Chromatogram in-line RPLC/ESI/QUAD/TOF MS (1D-LC/MS)




Max 400 counts
291.2232
y1
SNAP25b
Xenopus Laevis
99% Confidence
gi 27448109
MS/MS of 729.9 precursor ion
145.1053
432.2260
b3
ADQLADESLESTR
b2
a1
b1
y2
y3
a3
y1-NH3
b3-H2O
b4
b5
y4
x5
y9
a2
y2-NH3
y3-H2O
b6
y6
Intensity, counts
m/z, amu


Max 390 counts
SNAP25b; Xenopus Laevis Error Factor (EF)<2
Avg area ratio value for 2 peptides
*Upregulation pattern consistent with gels
114
115
116
117
Reporter Region
Control
PTU
R=1.35
Perchlorate
R=1.30
Methimazole
R1.38
Intensity, counts
m/z, amu

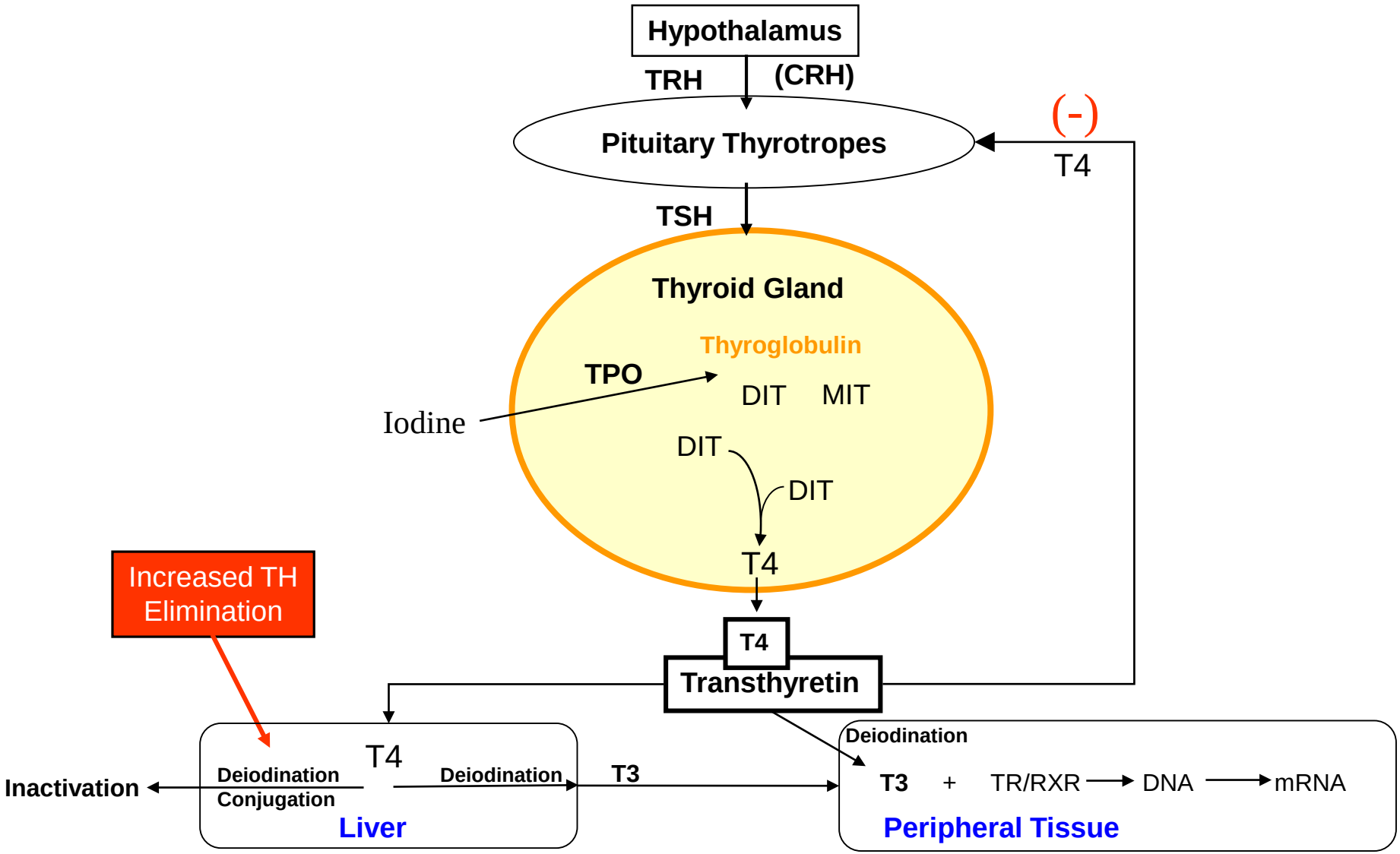
Up-Regulated Proteins

Protein id Xenopus laevis **Validated by iTRAQ*	*General Biological Function*
***SNAP25b**	Membrane fusion
***Hnrpa1-prov**	Gene expression modulation
Hnrpk-prov	Gene expression modulation
***Eno-1-prov**	Metabolism
***aldolase**	Metabolism
***Hspa5-prov**	Stress response
XNIF	Neuronal filament protein

Down-Regulated Proteins

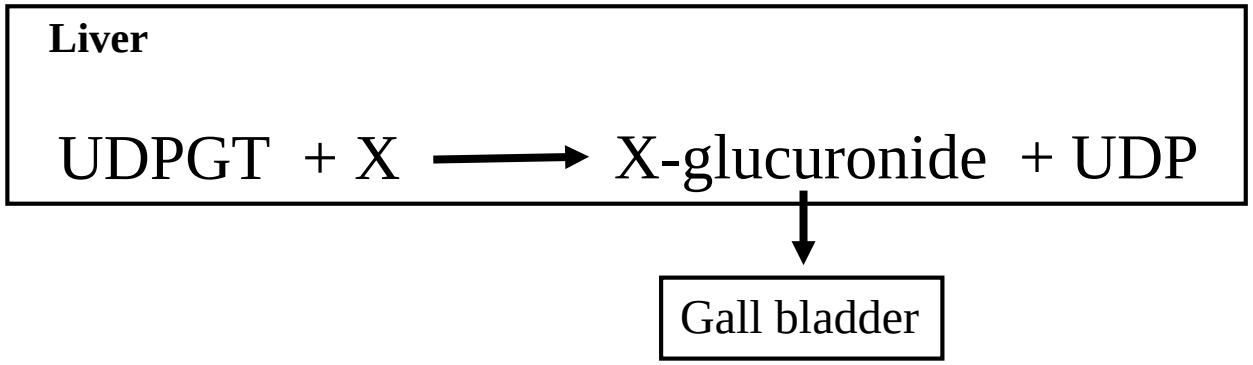
Protein id Xenopus laevis **Validated by iTRAQ*	*General Biological Function*
***Ywhaq-prov**	Gene encoding control
MGC64423	Gene encoding control
***Hb T3/T4**	Oxygen Transport
Calbindin D	Metamorphosis
DRP-3 neural	Axonal outgrowth/path
Alpha-Fodrin	Cytoskeletal support
Flotillin 1C	Raft protein
Vcp-prov	Cell division
Cofilin1	Structural support

Thyroid Disruption Pathways



Thyroid Axis Disruption by UDPTG Inducers

- Jasim Chowdhury - NRC
- UDP-glucuronosyltransferase (UDPGT)
- Conjugates endobiotics and xenobiotics for elimination



- 35 known UDPGT isoforms across animal species
- *Xenopus* isoforms are not yet known
- 6 UDPGT genes are identified for testing

- Model UDPGT Inducer: Phenobarbital

Effects of phenobarbital on larval development

Final tadpole stages

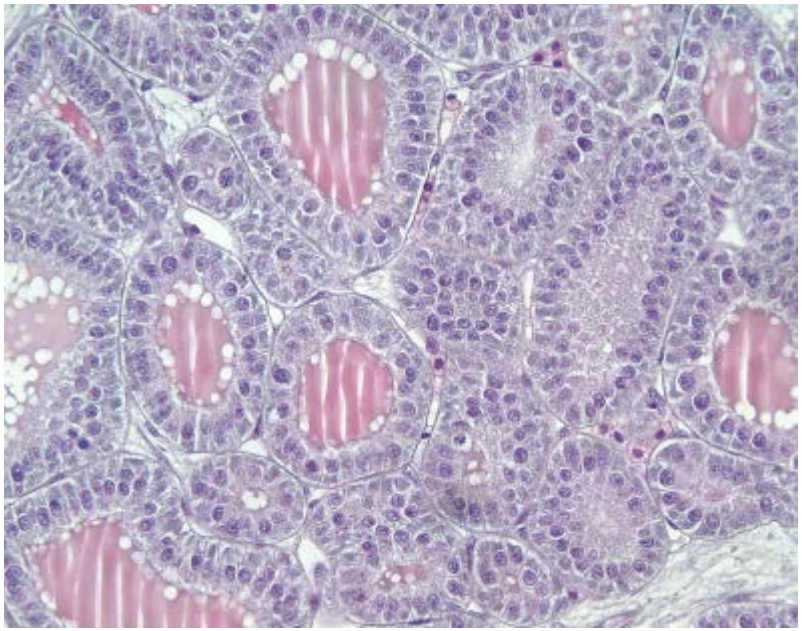
Phenobarbital (mg/L)	57	58	59	60	61	62	63
0			10	51	11	18	10
500			28	53	17	2	
1000	3		25	69	3		
1500		9	38	53			

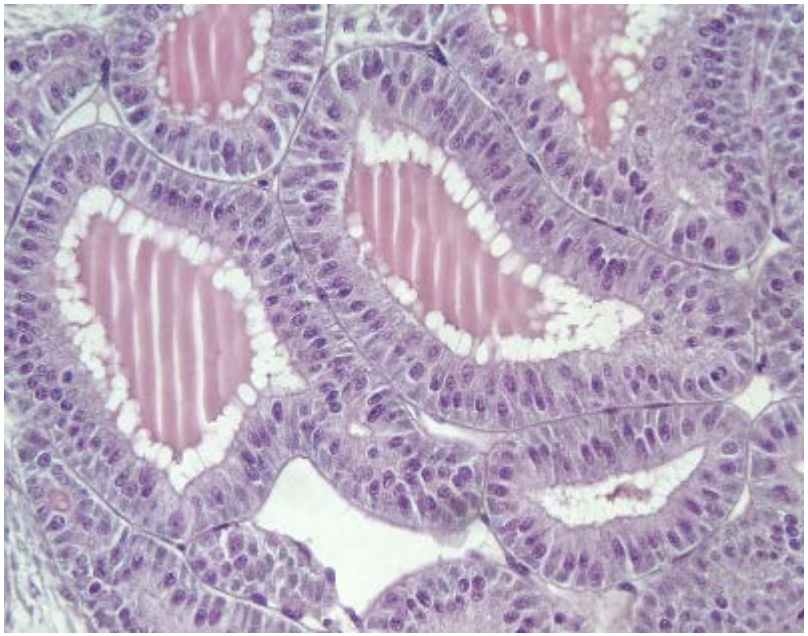
Proportion in stage

Effects of phenobarbital on thyroid glands (stage-60 tadpoles)

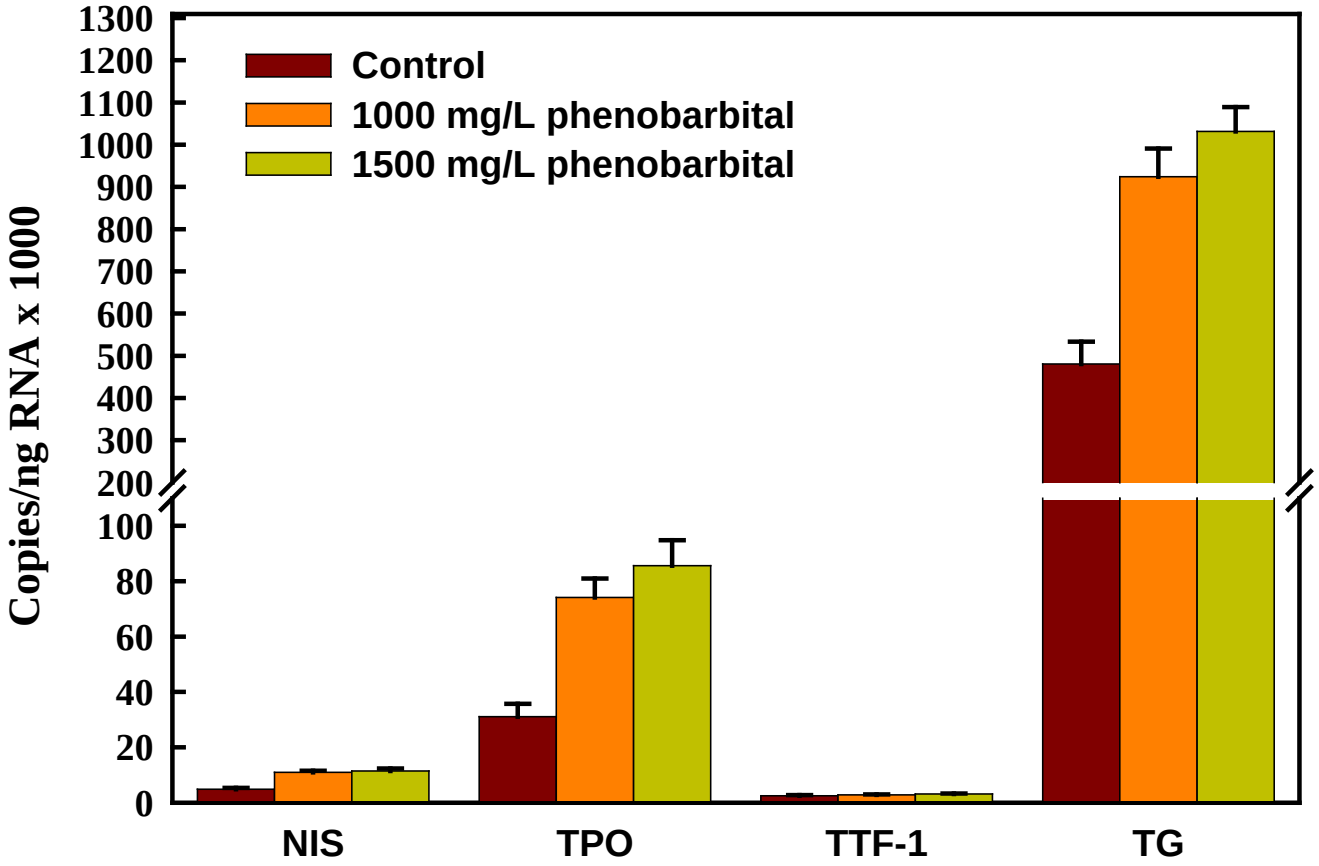
Histology

Control **1500 mg/L**

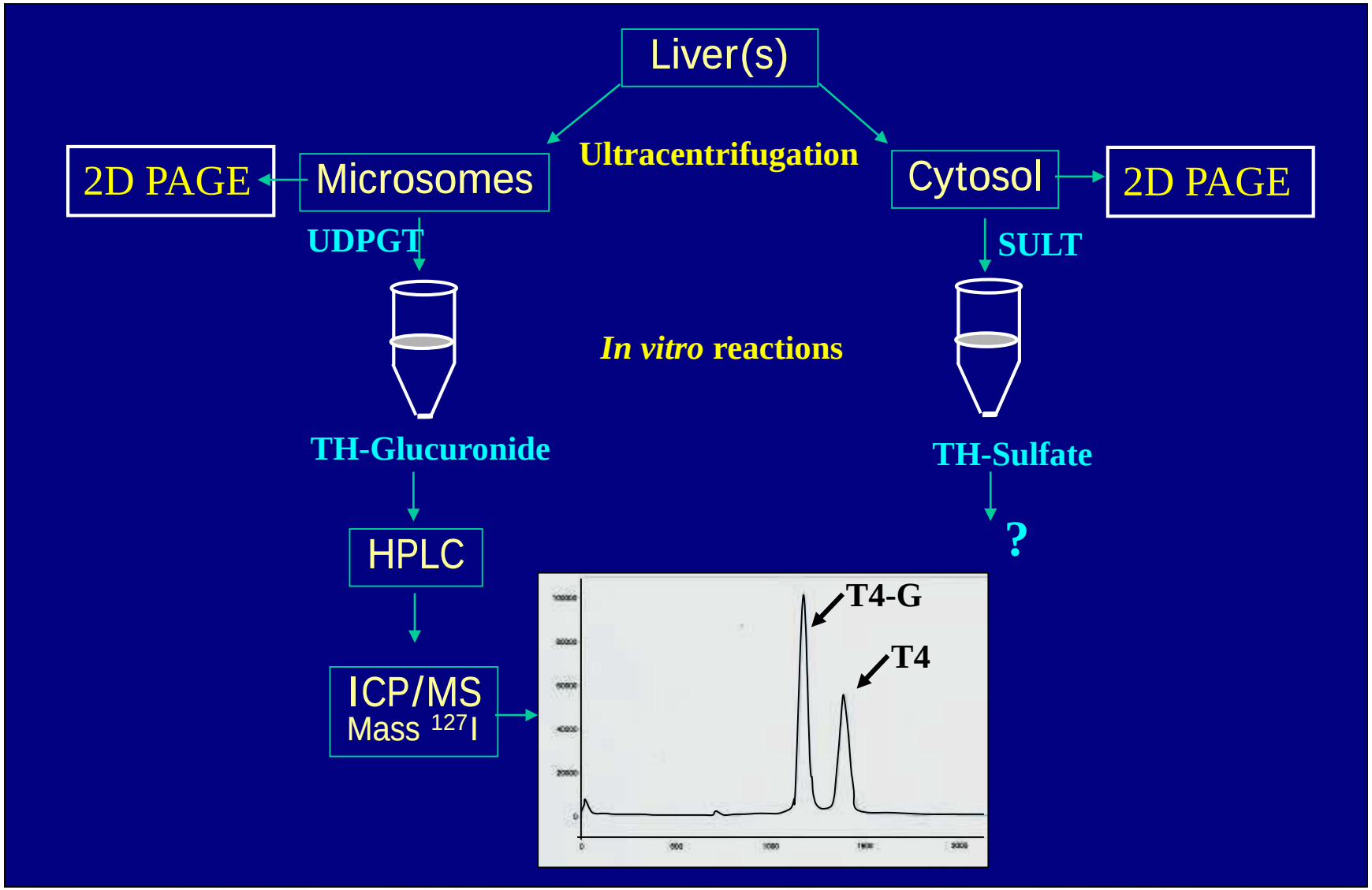




Thyroid genes in stage-60 tadpoles



Overview of enzyme assays for TH conjugates



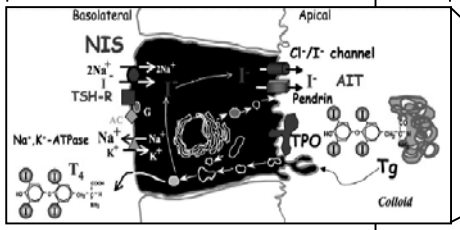
Conclusions

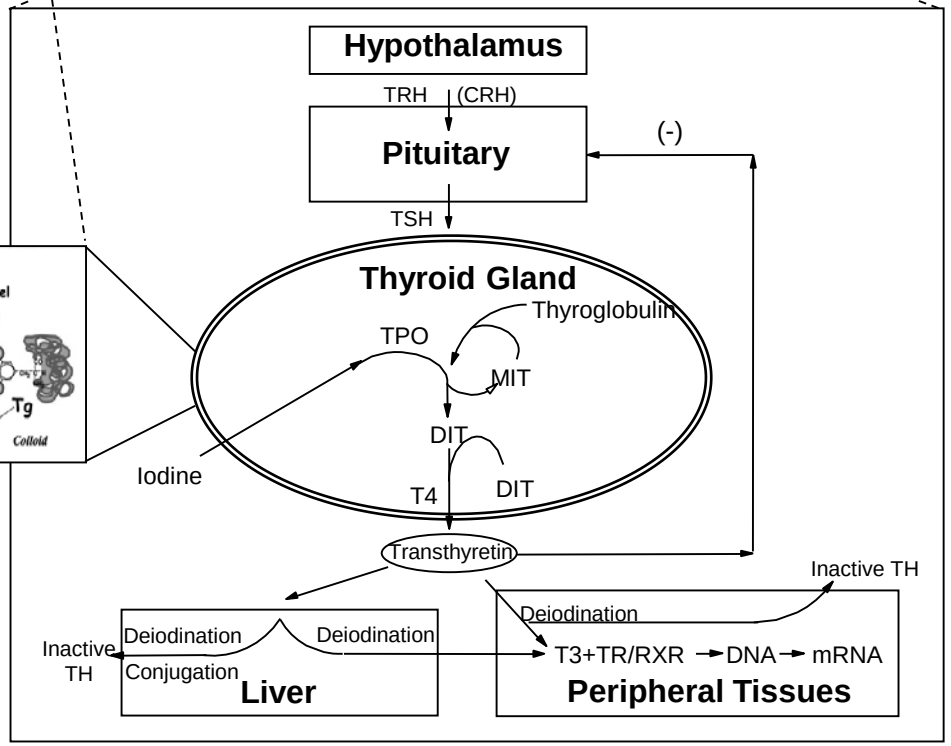
- A short term assay can be developed for screening chemicals
- *X. laevis* respond to model thyroid pathway modulators in a similar manner to higher vertebrates
- A multi-endpoint strategy is useful for diagnostic information on chemical effects - linking biochemical/molecular endpoints with whole organism effects

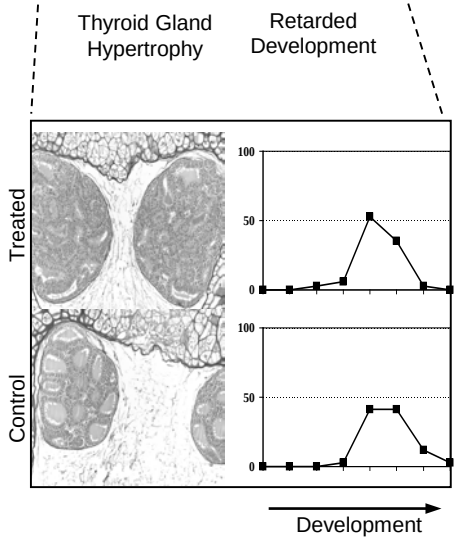
Predictive Toxicology / Thyroid Systems Model

QSAR and *in vitro* Models → **Systems Model** → **Organismal Outcomes**

Thyroid Follicular Cell







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